Stellenbosch University

FREE LIGHT CHAINS IN PATIENTS WITH HIV: ESTABLISHING LOCAL REFERENCE RANGES AND THEIR ASSOCIATION WITH STAGE OF DISEASE, CHRONIC ANTIGEN STIMULATION AND THE EFFECT OF HAART

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DECLARATION

I, the undersigned, hereby declare that the work contained in this assignment is my original work and that I have not previously submitted it, in its entirety or in part, at any university for a degree.

Signature: .....……..... **Date: ...…….....**

March 2012

ABSTRACT

Background: Serum free light chains (FLC) are associated with imbalances in heavy and light chain production. Abnormal FLC ratios have been associated with risk of progression in certain diseases. Automated assays are available for their determination and they are used in the follow-up and management of patients with monoclonal gammopathies. Acceptable imprecision, specificity, accuracy and reproducibility between reagent batches is required to prevent under- or overestimation. Method validation is a standard process in every good laboratory to judge the acceptability of a new method. Reference intervals have been established in an older population, but it was considered important to verify these in our population. HIV is associated with B-cell dysfunction. As B-cell abnormalities are associated with disorders leading to monoclonal gammopathies, we postulated that the FLC levels and FLC ratio would be abnormal in HIV infected individuals.

Methods and materials: Controls and pooled patient samples were used for the method validation study which included imprecision studies, linearity, recovery and interference studies, and method comparison studies, the latter compared our method to the same method used in another laboratory. For the reference interval study, blood was obtained from 120 healthy subjects. The following blood tests were performed: total protein, IgG, IgA, IgM, creatinine, protein electrophoresis, kappa FLC and lambda FLC. Using the kappa and lambda FLC results, a FLC ratio was determined. Three hundred and sixty-nine HIV positive subjects were then studied. The same tests were performed, as well as $CD4^+$ counts and viral loads on the majority of them.

Results: For the method validation study, precision, linearity and recovery was acceptable. Minimal interference was observed with haemolysis, lipaemia, bilirubin and rheumatoid factor. Our method showed comparable performance with the established method. For the reference interval study, all the creatinine values were normal, as were serum protein values. The serum protein electrophoreses were independently reviewed by 3 pathologists. Most were normal, with a few polyclonal increases seen, but no definite monoclonal bands. The 95% reference intervals for FLC's as well as the FLC ratio were not statistically significantly different to the manufacturer's recommendations. When examining the HIV positive study population, we found

that FLC and FLC ratio were influenced by markers of HIV disease severity, such as $CD4^+$ count, IgG, viral load, use of antiretroviral treatment and abnormal serum protein electrophoreses.

Conclusion: The validation study of FLC showed excellent precision, acceptable bias, good linearity, good recovery and minimal interference, allowing routine introduction of the test. The 95% reference intervals obtained for our population were slightly higher than those recommended by the manufacturer. However, as most of the values fell within the manufacturer's limits, we could accept the manufacturer's recommended cut-offs. We found that FLC levels were definitely influenced by markers of HIV disease severity in our population and we postulate that they may be of use for follow-up of patients with HIV.

ABSTRAK

Agtergrond: Serum vry ligte kettings (VLK) word geassosieer met ‘n wanbalans van ligte en swaar ketting produksie. Abnormale VLK ratios is geassosieer met ‘n risiko van verloop in sekere siektes. Geoutomatiseerde laboratorium toetse vir VLK is beskikbaar vir hul bepaling en word gebruik om pasiënte met monoklonale gammopatieë op te volg en te behandel. Aanvaarbare impresisie, spesifisiteit, akkuraatheid en herhaalbaarheid tussen reagens besendings is belangrik om onder- of oorbepaling te verhoed. Metode validasie is ’n standaard proses in elke goeie laboratorium om die aanvaarbaarheid van ’n nuwe metode te bepaal. Verwysingswaardes is al bepaal in ’n ouer populasie. Ons het besluit om die verwysingswaardes in ons populasie te bepaal. Mens-immuungebrekvirus (MIV) word geassosieer met B-sel disfunksie. Omdat B-sel abnormaliteite geassosieer word met afwykings wat tot monoklonale gammopatieë lei, het ons gepostuleer dat die VLK vlakke en VLK ratio abnormaal sal wees in MIV geïnfekteerde persone.

Metodes en Materiale: Kontroles en pasiënt monsters is gebruik vir die metode validasie studie wat impresisie studies, lineariteit, herwinning, inmenging en metode korrelasie studies ingesluit het. In laasgenoemde geval is ons metode met dieselfde metode van ’n ander laboratorium vergelyk. Vir die verwysingswaardes studie is 120 gesonde persone se bloed gebruik. Die volgende toetse is bepaal: totale proteïen, IgG, IgA, IgM, kreatinien, proteïen elektroferese, kappa en lambda VLK. Die VLK ratio is bepaal deur die kappa en lambda resultate te gebruik. Driehonderd nege en sestig MIV-positiewe pasiente is gebruik vir die studie. Dieselfde toetse was gedoen, asook $CD4^+$ tellings en virale ladings op die meerderheid van pasiente.

Resultate: Vir die metode validasie studie, was presisie, lineariteit en herwinning aanvaarbaar. Minimale inmenging van hemolise, lipemie, bilirubien en rumatoïede factor is waargeneem. Ons metode het goed gekorreleer met die bepaalde metode. Die serum kreatinien en serum totale proteïen waardes was normaal tydens die verwysingswaardes studie. Die serum proteïen elektroferese was onafhanklik beoordeel deur 3 patoloë. Die meeste was normaal met enkele poliklonale verhogings, maar geen definitiewe monoklonale bande nie. Die 95% verwysings intervalle vir VLK en VLK ratio het nie statisties betekenisvol verskil van die vervaardiger se aanbevelings nie. In die studie van die MIV-positiewe studie populasie, het ons gevind dat VLK

en VLK ratio beïnvloed word deur merkers van ernstige MIV siekte, soos $CD4^+$ telling, IgG, virale lading, die gebruik van antiretrovale medikasie en abnormale serum proteïen elektroferese.

Gevolgtrekking: Die validasie studie van VLK het uitstekende presisie, aanvaarbare partydigheid, goeie lineariteit, goeie herwinning en minimale inmenging gewys, wat die roetine instelling van die toets toegelaat het. Die 95% verwysingsintervalle wat vir ons populasie bepaal is, was effens hoër as die vervaardiger se aanbeveling. Die meeste van die waardes het egter binne die vervaardiger se limiete geval, dus kon ons die vervaardiger se afsnypunte aanvaar. Ons het gevind dat VLK vlakke definitief beïnvloed word deur merkers van die ernstigheidsgraad van MIV siekte in ons populasie en ons postuleer dat VLK van waarde kan wees met die opvolg van MIV pasiente.

TABLE OF CONTENTS

Appendix IX: Correlation IgG to FLC

Appendix X: Relationship between Stage of Disease and FLC

Appendix XI: Relationship between ART use and FLC

Appendix XII: Relationship between Immunofixation for Abnormal Serum Protein Electrophoresis and FLC

LIST OF ABBREVIATIONS AND SYMBOLS

AIDS	Acquired Immunodeficiency Virus
ARC	AIDS-related complex
ART	Antiretroviral therapy
CLIA	Clinical laboratory improvement amendments
CLSI	Clinical Laboratory and Standards Institute
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
EBV	Ebstein Barr virus
ELISA	Enzyme linked immunosorbent assay
FLC	Free light chain
HAART	Highly active antiretroviral treatment
Hb	Haemoglobin
HIV	Human Immunodeficiency Virus
HSRC	Human Science Research Council
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LAS	Lymphadenopathy syndrome
LCMM	Light chain multiple myeloma
MGUS	Monoclonal gammopathy of unknown significance
MIV	Mens-immuungebreksvirus
MM	Multiple myeloma

NHL	Non-Hodgkin's lymphoma
NK-cells	Natural Killer cells
NSMM	Nonsecretory multiple myeloma
PEL	Primary effusion lymphoma
RNA	Ribonucleic acid
RF	Rheumatoid factor
SLE	Systemic Lupus Erythrematosus
SMM	Smouldering multiple myeloma
TB	Tuberculosis
TBH	Tygerberg Hospital
TNF	Tumour necrosis factor
VLK	Vry ligte kettings
WHO	World Health Organization

α	Alpha
β	Beta
γ	Gamma
κ	Kappa
λ	Lambda

®	Registered trademark
™	Trademark

LIST OF FIGURES

Section I: Literature Review

Section III: Results

LIST OF TABLES

Section I: Literature Review

Section III: Results

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INTRODUCTION

Human Immunodeficiency Virus (HIV) is a retrovirus belonging to the genus Lentiviruses. Currently, HIV infection is a worldwide epidemic. In poor countries with a high prevalence of HIV, such as South Africa, with an estimated 5.3 million people living with HIV/Acquired Immunodeficiency Syndrome (AIDS), the cost implication of unnecessary investigation of patients may have a significant impact on health care spending and allocation of resources (Abdool Karim et al. 2009). South Africa is facing an enormous challenge with its HIV and tuberculosis (TB) epidemics. Today there are an estimated 38 million people living with HIV or AIDS and over 5 million of those people are living in South Africa. This is the highest percentage of people in any single country that is HIV positive, according to a study done by the United Nations in 2010. The previous count made in 2009 of Eastern and Southern Africa points to 7.7 million people in need of antiretroviral therapy (ART); and the estimated number receiving treatment are 3.2 million, which is a mere 41% (www.unaids.org).

Infection with HIV is associated with a gradual decline of $CD4^+$ T-cells as well as several B-cell abnormalities. The latter includes polyclonal activation, hypergammaglobulinaemia, auto-immune phenomena, defective response to antigen stimulation and the occurrence of AIDS-related lymphomas (De Milito. 2004). Monoclonal proteins have also been noted with increased frequency on serum protein electrophoresis in patients with HIV (Heriot K et al. 1985). This and the association of HIV with non-Hodgkins lymphoma (NHL) may be due to B-cell dysfunction in these patients.

SECTION I: LITERATURE REVIEW

1.1 HIV INFECTION

The HIV virus targets the immune system by attacking $CD4^+$ T-cells. The virus inserts its genetic material into the $CD4^+$ T-cell's DNA and replicates. The host $CD4^+$ T-cells eventually die and the body's ability to defend itself against disease decreases until eventually, the $CD4^+$ T-cells decrease to such an extent, that the patient develops AIDS. The virus copies its genetic material into the $CD4^+$ T-cells with an increasing level of error. Thus the HIV replicates with a high rate and mutates at a high speed. Additionally, the envelope that contained the HIV particle consists of the same material as human cells, thus making it difficult for the immune system to distinguish between virus particles and healthy cells (www.news.bbc.co.uk).

HIV infection can be divided into 3 different phases as shown in figure 1.1:

1. The acute phase

The acute phase of HIV occurs six to twelve weeks after infection and lasts until anti-HIV antibodies are detectable in the blood. HIV infection is characterized by a deterioration of the cellular immune system with a steady decrease in $CD4^+$ T-cells leading to various clinical symptoms (Vergis et al. 2000). The loss of $CD4^+$ T-cells correlates with viral load. The degree of immunodeficiency associated with HIV infection correlates with $CD4^+$ T-cell counts and absolute $CD4^+$ counts are used to determine when to initiate highly active antiretroviral treatment (HAART). Recently it has been reported that the majority of this memory $CD4^+$ T-cell loss occurs in the gastrointestinal tract in the first few weeks after infection, as this is where 80% of the $CD4^+$ T-cell population is found (Brenchley et al. 2004). These $CD4^+$ T-cells express the CCR5 HIV coreceptors which enable entry of HIV into the host cell. The loss of gut memory T-cells leads to loss of mucosal integrity and microbial translocation to the systemic circulation (Douek D. 2007). This results in chronic immune activation, which is believed to contribute further to the progressive depletion of the remaining $CD4^+$ T-cells (Smith S. 2006; Appay and Sauce 2008; Virgin and Walker. 2010). Immune activation in chronic HIV infection

includes polyclonal B cell activation, increased T-cell turnover, increased activated T-cells and increased cytokines and other inflammatory mediators. This can help with restoration of memory $CD4^+$ T-cells and immunocompetence, but unfortunately also leads to lymph node fibrosis, retention of effector T-cell in lymph nodes, thymic dysfunction, clonal exhaustion, drainage of memory T-cell pools, and generation of more targets for HIV to permit ongoing HIV replication (Douek D. 2007; Sodora and Silvestri. 2008).

2. The latent period

The immune response to the infection is able to control viral replication to a certain extent and viral particles decrease in the blood stream. The patient enters a clinically asymptomatic (or latent) phase with only few viral particles detectable in the bloodstream or peripheral blood lymphocytes and the $CD4^+$ count is only slightly decreased. During the latent phase, there may be no clinical manifestations, except for a generalized lymphadenopathy. The lymphoid tissue serves as a major reservoir for HIV, with the follicular dendritic cells in this tissue filtering and trapping free virus and infected $CD4^+$ T-cells. This leads to disruption of the lymph node architecture and release of HIV (Douek. 2007; Brenchley et al. 2004; Swingler et al. 2008).

3. Onset of disease – AIDS

This occurs between 3-15 years after initial infection. The virus can no longer be controlled as $CD4^+$ T-cells are destroyed and leads to loss of immune competence.
The humoral immunity also decreases with B-cells exhibiting an increase in markers of activation and proliferation. (Moir et al. 2004; Swingler et al. 2008). These B-cells undergo terminal differentiation leading to an increase in immunoglobulin secretion and polyclonal hypergammaglobulinaemia. However, these antibodies are nonspecific, which explains why the patients are still at risk of bacterial infections (Moir et al 2001).

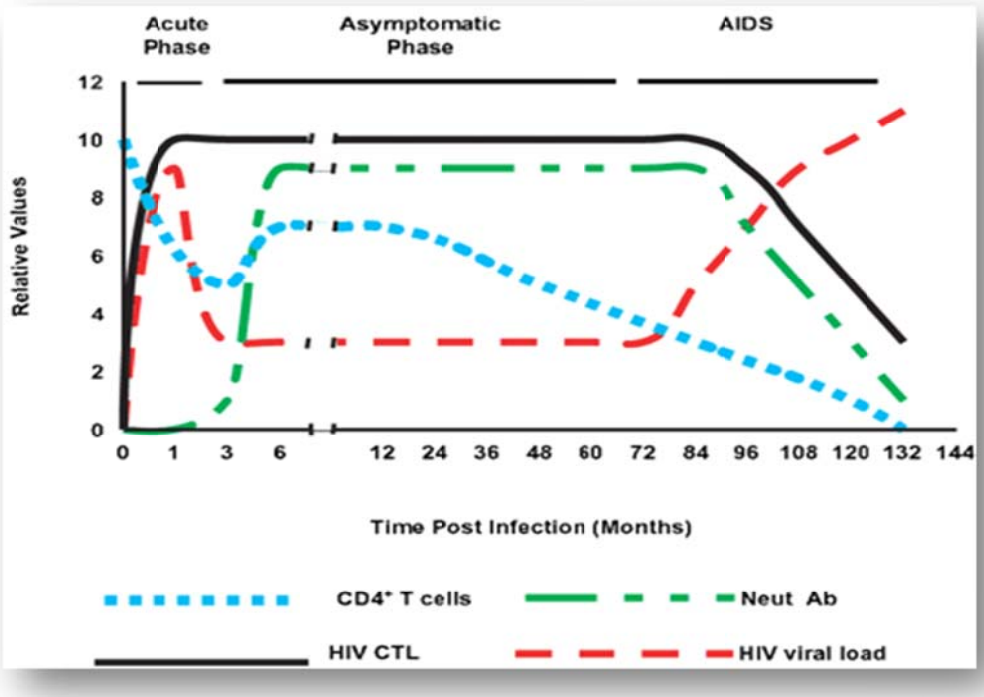

Figure 1.1 The 3 phases of HIV infection

1.2 B-CELL ABNORMALITIES IN HIV

HIV infection leads to abnormalities of both cellular and humoral immune systems (Moir et al. 2009; Appay and Sauce. 2008). In addition to the gradual decline in $CD4^+$ T-cells, several abnormalities in B-cells have been described.

Generalized B-cell hyperactivity is a widely accepted feature of HIV pathogenesis and disease progression and is characterized by hypergammaglobulinaemia (Moir et al. 2001; Notermans et al. 2001; Shearer et al. 2000; Moir et al 2008; De Milito. 2004) , increased polyclonal B-cell activation (Lane et al. 1983; Moir et al. 2001; Shirai et al. 1992; Moir et al. 2008), increased cell turnover (Moir et al. 2004; Kovacs et al. 2001), increased expression of activation markers (Malasspina et al. 2003; van der Meijden et al. 1998), increased differentiation of B-cells to plasmablasts (Moir et al. 2001; Moir et al. 2004; Nagase et al. 2001; Conge et al. 1998; Moir et al. 2008), increased production of

autoantibodies (Shirai et al. 1992; Haynes et al. 2005; Ng et al. 1996; Moir et al. 2008; de Milito. 2004) and an increase in the frequency of B-cell malignancies (Martinez Maza et al. 2002; Moir et al. 2008). Monoclonal proteins have also been noted with increased frequency on serum protein electrophoresis in patients with HIV (Jansen van Vuuren et al. 2010).

A probable cause of abnormal clonal B-lymphocyte proliferation in patients with HIV infection is the hyperstimulated state where more B-cells spontaneously secrete immunoglobulins and fewer B-cells are in the resting state (Lane et al. 1983; Caggi et al. 2008). There are several possible causes for this polyclonal B-cell activation. Firstly, certain infections in healthy people may result in the development of paraproteins because of hyperstimulation and subsequent immunoglobulin production. HIV infected patients have other infective processes that resulted in higher immunoglobulin production, but may not be detected clinically (Schnittmann et al. 1986). HIV infected patients are known to have a higher incidence of infections due to certain viruses, including Epstein-Barr virus (EBV), herpes virus, hepatitis B virus and cytomegalovirus (CMV). These DNA viruses are capable of inducing B-cell activation (Schnittmann et al. 1986). Secondly, B-cell activation may be secondary to alteration of regulatory T-cell influences (de Milito. 2004; Caggi et al. 2008). Thirdly, B-cell activation in HIV infection may result from the virus actually infecting B-cells and acting as a polyclonal activator, analogous to EBV infection of B-cells (Schnittmann et al. 1986). This has recently been shown to occur in vitro. An autonomous clone could arise from virus-activated B-cells if there was integration of the virus near a genetic enhancer element or a proto-oncogene which would induce cellular transformation (Crapper et al. 1987).

HIV–induced immune dysfunction is not only related to B-cells, but also to other components of the immune system such as the low $CD4^+$ T-cells with the loss of immune function (Lane et al. 1983). In untreated patients, HIV infection leads to chronic immune activation through indirect systemic effects or bystander effects of ongoing replication. Bystander effects have been described for $CD4^+$, $CD8^+$ T-cell, natural killer (NK) cells and B-cells (Grossman et al. 2006; Sodora and Silvestri. 2008; Moir and Fauci. 2009).

In healthy individuals most B-cells in the periphery are resting naïve B-cells or memory B-cells expressing either switched or unswitched antibody isotypes (IgG, IgE and IgA, or IgM and IgD respectively)(Moir and Fauci. 2009). In HIV infected patients, several additional B-cell subpopulations, which are not normally present in the peripheral blood of uninfected individuals, can make up significant fractions of the total B-cell population including: immature transitional B-cells, exhausted B-cells, activated mature B-cells and plasmablasts (Moir and Fauci. 2009).

The effects of ongoing HIV replication on B-cells are thought to reflect a combination of direct interactions of B-cells with the virus and indirect interactions that are associated with a wide range of systemic alterations (Moir and Fauci. 2009). Direct interactions between HIV and B-cells were reported years ago, although there is little evidence that HIV can productively replicate in B-cells in vivo (Schnittman et al. 1986). There is evidence that HIV binds to B-cells in vivo via the complement receptor CD21(Moir and Fauci. 2009), which is expressed on most mature B-cells and complement proteins bound to HIV virions that circulate in vivo (Kacani et al. 2000; Moir et al. 2000). These immune-complex-based interactions might provide stimulating signals to B-cells, although this is of low frequency. This B-cell-HIV virion interaction most likely facilitates cell-to-cell transmission of HIV (Malaspina et al. 2002). A similar mechanism of HIV interaction has been suggested for follicular dendritic cells which also express CD21 and might function as a long-lived extracellular reservoir for HIV even in the presence of effective ART (Rappocciolo et al. 2006).

Several cytokines and growth factors have been suggested to directly or indirectly trigger the activation of B-cells in HIV patients, namely interferon-α (IFN) (Mandl et al. 2008), tumour necrosis factor (TNF) (Rieckman et al. 1991), interleukin-6(IL 6), IL-10 (Weimer et al. 1998), CD10 ligand (Muller et al 1998) and B-cell activating factor (He et al. 2006). These factors are increased during HIV infection and are thought to be associated with B-cell hyperactivation in these patients.

Another indirect effect of HIV on B-cells is **HIV-induced lymphopaenia.** In most untreated patients, HIV infection leads to a loss of $CD4^+$ T-cells. T-cell homeostatic cytokine IL-7 is dysregulated in HIV-infected patients with advanced HIV-associated disease. Increased serum levels of IL-7 were associated with decreased numbers of $CD4^+$ T-cells (Rappocciolo et al. 2006: He et al. 2006; Moir and Fauci. 2009). IL-7 can induce the proliferation of human B-cell precursors (Le Bien and Tedder. 2008). A high viral load and low $CD4^+$ T-cell counts are thus associated with increased serum levels of IL-7 and increased numbers of immature transitional B- cells (Malaspina et al. 2006). A similar association between increased serum levels of IL-7 (Moir and Fauci. 2008), B-cell immaturity and decreased $CD4^+$ T-cell counts was observed in patients with non-HIV-related idiopathic $CD4^+$ T-cell lymphopaenia, which suggests that HIV-induced $CD4^+$ T-cell lymphopaenia and not HIV viraemia itself drives the expansion of immature transitional B-cells in HIV infected patients (Conge et al. 1998).

The third indirect effect of HIV on B-cells is HIV-associated B- cell exhaustion. The loss of CD21 on peripheral blood cells is a reliable marker of ongoing HIV replication and disease progression (Moir and Fauci. 2008). $CD21^{low}$ B-cells constitute a heterogeneous population of cells in infected HIV patients, one fraction of the $CD21^{low}$ B-cell compartment is made up of CD27 B-cells that have undergone HIV-induced activation and differentiation to plasmablasts (He et al. 2006) and another fraction is made up of immature transitional CD10 B-cells that are over-represented as a result of HIV-induced T-cell lymphopaenia (Brenchley et al. 2006). A large proportion of $CD21^{low}$ B-cells in HIV-viraemic patients does not fit into either of these fractions and it is believed that these B-cells constitute an exhausted B-cell subpopulation (Moir et al. 2008). Exhausted B- cells refers to virus- specific immune cells that have lost their function due to the chronic nature of viral infection (Day. 2006; Trautmann et al. 2006; Virgin and Walker 2010).

There is strong evidence to suggest that memory B-cells are exhausted in the peripheral blood of HIV viraemic patients (Moir and Fauci. 2008; Moir and Fauci. 2009; Bussmann et al. 2009). As HIV specific responses increase in B-cells that show signs of functional

exhaustion, such dysregulation might contribute to the inefficiency of the antibody response against HIV in infected patients. Chronic HIV viraemia is associated with the expansion of several aberrant B-cell subpopulations including immature transitional, hyperactivated and exhausted, which can contribute to various B-cell dysfunctions (Ehrhardt et al. 2008; Wherry et al. 2007; Shin and Wherry. 2007).

Some B-cell abnormalities associated with HIV-replication induced immune-cell activation can be reversed by ART, whereas others, particularly loss of memory B-cells persist, even after several years of effective ART (Morris et al. 1998). The B-cell abnormalities that decrease with ART include hypergammaglobulinaemia and HIV-specific and HIV non- specific B-cell responses as measured by the number of B-cells that spontaneously secrete high levels of immunoglobulins (Moir et al. 2001; Notermans et al. 2001; Amman et al. 1984; Nilssen et al. 2004).

B-cells of HIV-infected patients express high levels of activation markers and studies have shown that these activation markers are normalized by ART (Moir et al. 2004; Riekman et al. 1991). One of the consequences of HIV- induced chronic immune cell activation is increased cell turnover with cell proliferation and cell death. Studies have shown an increased turnover of $CD4^+$ and $CD8^+$ T-cells, as well as of NK cells and B-cells during HIV infection. This increased turnover is reversed by ART (Kovacs et al. 2001; de Boer et al. 2003). In most HIV infected individuals, the initiation of ART leads to a gradual increase in $CD4^+$ T-cell counts and a decrease in $CD8^+$ T-cell counts (Ribeiro. 2007). Several studies have shown that B-cell numbers are decreased in HIV infected individuals (Shearer et al. 2000; Meira et al. 2005), however with ART, B-cell numbers increase and B-cell dynamics in response to infection are more closely related to those of $CD4^+$ rather than $CD8^+$ T- cells (Moir et al. 2008; Le Guillou-Guetemette. 2006). An increased rate of B-cell death during viral replication may also contribute to the loss of B-cells (Ho et al. 2006; Moir et al. 2004).

Although most B-cell defects in HIV infection can be reversed by ART, one important exception is the loss of memory B-cells and the decrease in memory B-cell function (De

Milito. 2004; Caggi et al. 2008). CD27 is a marker that is used to define memory B-cells. Studies have shown that although ART leads to decreased numbers of $CD27^+$ activated B-cells and plasmablasts, the increase in number of $CD27^+$ resting memory B-cells after treatment occurs slowly and remains incomplete. (Moir et al. 2008; Chong et al. 2004; D'Orsagna et al. 2007; Jacobsen et al. 2008; De Milito et al. 2001).

Early initiation of ART appears to reverse an important consequence of chronic HIV infection, namely the of IgM^+ memory B-cells (Titanji et al. 2005; Moir and Fauci. 2008).

1.3 MONOCLONAL PROTEINS

A paraprotein or monoclonal or M-peak refers to an immunoglobulin molecule produced by a clone of plasma or B-cells and is usually detected as a band in the alpha to gamma area on protein electrophoresis of serum or urine.

Monoclonal immunoglobulins are found in a number of disorders, benign and malignant. Best known are the plasma cell dyscrasias, multiple myeloma (MM), amyloidosis, plasmacytomas and B-cell lymphoproliferative disorders such as Waldenströms macroglobulinaemia, small lymphocytic lymphoma and other B-cell lymphomas (Pontet. 2005).

Other disorders associated with monoclonal proteins include auto-immune disease (systemic lupus erythrematosus (SLE), Sjögren's syndrome and diabetes), HIV and other serious infections (septicaemia, TB and meningitis), chronic liver disease (cirrhosis, hepatitis), malignant or benign tumors and other haematological neoplasms (myeloproliferative disorders, Hodgkin's disease) (Pontet. 2005).

1.3.1 Premalignant plasma cell disorders:

1.3.1.1 Monoclonal gammopathy of undetermined significance (MGUS)

MGUS is a premalignant plasma cell proliferative disorder, the feature of which is the presence of monoclonal immunoglobulin or M protein in the serum. MGUS is found in 3.2% of the general population 50 years and older and 5.3% of those older than 70 years (Kyle et al. 2006). Patients with MGUS have a serum M protein of less than 30g/L, bone marrow plasma cells of less than 10%, and no anaemia, hypercalcaemia, lytic bone lesions or renal failure that would be a sign of a malignant plasma disorder. MGUS is asymptomatic, but does progress to MM or related malignancy at a rate of 1% per year. Persons with MGUS must be followed-up annually. Risk factors were very difficult to identify for the progression of MGUS. The type and size of M protein were the only predictive risk factor of progression (IgM and IgA) (Kyle et al. 2006). In a study by Cesana *et al* it was found that a bone marrow plasma cell count of 6% to 9% had twice the risk of progression as compared with bone marrow plasma cell of less than 5% (Cesana et al. 2002).

1.3.1.2 Smouldering MM (SMM)

SMM was first reported in 1980 and is an asymptomatic premalignant plasma cell disorder with the potential risk of progression to symptomatic MM. The definition for SMM is as follows: a serum M protein level greater than or equal to 30g/L and/or bone marrow plasma cells greater than or equal to 10%, no anaemia, hypercalcaemia, renal failure, or lytic bone lesions, no end organ damage or symptoms of myeloma (Dispenzieri et al. 2008). It is important that SMM be distinguished from the clinically aggressive MM and the more benign MGUS with much lower risk of progression (Kyle et al. 2002). In a study by Dispenzieri *et al* the risk of progression from SMM to MM was determined if the patients with SMM meet both the M protein and bone marrow criteria. After 10 years 77% of the patients with plasma cells greater or equal to 10% and M protein greater than or equal to 30g/L, 64% of the patients with plasma cells greater or equal to 10% and M

protein less than 30 g/L and 33% of the patients with plasma cells less than 10% and M protein was greater or equal than 30g/L progress to MM (Dispenzieri et al. 2008).

1.3.2 Malignant plasma cell disorders:

1.3.2.1 Multiple myeloma (MM)

MM is a haematological plasma cell proliferative malignancy characterized by the neoplastic proliferation of a single clone of plasma cells producing a monoclonal immunoglobulin. The clone proliferates in the bone marrow and produces osteolytic bone lesions, osteopaenia, osteoporosis and /or pathologic fractures. The monoclonal proteins may cause organ damage, often leading to renal insufficiency and /or renal failure. The mean age of diagnosis for multiple myeloma is 66 years with only 2% of patients younger than 40 years (Kyle et al. 2003).

The diagnosis of MM is based on the presence of 10% or more plasma cells in the bone marrow, monoclonal immunoglobulins in the serum or urine and a monoclonal band of 30g/L or more on serum protein electrophoresis with related organ or tissue impairment such as renal failure, hypercalcaemia, anaemia and symptoms of bone lesions (Kyle et al. 2003, Smith et al. 2006).

1.3.2.2 Light chain MM

LCMM has the clinical features of MM. Bence Jones protein in urine protein electrophoresis is positive with the absence of intact monoclonal immunoglobulins in serum protein electrophoresis (Bridgen and Webber. 2000).

1.3.2.3 Nonsecretory MM (NSMM)

NSMM has the same symptoms of MM, but the tumour plasma cells contain no detectable immunoglobulins. There are no monoclonal bands in serum and urine protein electrophoresis (Dreicer and Alexanian. 1982; Drayson et al. 2001).

1.3.2.4 Intact Immunoglobulin MM

Patients with intact immunoglobulin MM have a monoclonal band in serum protein electrophoresis and no bands in urine protein electrophoresis (Blade and Kyle. 1999).

1.3.3 Waldenströms Macroglobulinaemia

Waldenströms macroglobulinaemia is a low tumor burden lymphoproliferative disorder that is associated with the production of monoclonal IgM. It is 5-10% as frequent as myeloma and the mean age of presentation is 65 years with a mean survival of five years and 20% of the patients live more than 10 years. Patients have high concentrations of IgM with infiltration of the bone marrow, spleen, liver and lymph nodes. Serum IgM quantification is important for diagnosis and monitoring (Owen et al. 2003).

1.3.4 Amyloidosis

Amyloidosis is a protein conformation disorder. The main feature is the accumulation of monoclonal free light chains or their fragments as amyloid deposits in organs. Patients present with heart or renal failure, but the skin, peripheral nerves and other organs may also be involved. The mean age of presentation is 70 years and it is very rare before 40 years. The mean survival is 12 months. It is caused by a slow growing clone of plasma cells secreting monoclonal light chains, mainly the lambda (λ) type. Monoclonal protein is an important diagnostic feature and is found in serum and urine of most patients. Protein electrophoresis indicates a typical nephrotic pattern, with low albumin, elevated α2 - and low γ – fractions with no observable monoclonal peak. Immunofixation

electrophoresis shows polyclonal immunoglobulin in the γ - fraction and small monoclonal λ - protein in the ß/γ region. Urine electrophoresis shows a considerable amount of protein, particularly albumin with a small monoclonal spike. Immunofixation electrophoresis shows a monoclonal λ protein against a background of polyclonal kappa (κ) and λ light chains. The monoclonal band in both serum and urine is too small to quantify (Kyle and Gertz. 1995; Abrahams et al. 2003).

1.4 INCIDENCE OF MONOCLONAL PROTEINS IN HIV

Infection with HIV is associated with an increased frequency of monoclonal proteins on serum protein electrophoresis (Lefrere et al. 1993). The prevalence of monoclonal protein was higher in the earlier studies (Heriot et al. 1985; Crapper et al. 1987; Tubat-Herrera et al. 1993), with recent studies showing a much lower prevalence, probably due to ART(Jansen van Vuuren et al. 2010)

In 1985 Heriot *et al* performed serum and urine protein electrophoresis on agarose gel on 24 patients with clinical HIV infection, 9 of which had lymphadenopathy syndrome (LAS) and 15 with AIDS. Of these, 8/15 with AIDS and 6/9 patients with LAS had paraproteins on serum protein electrophoresis. Twelve of the patients had IgG κ type paraprotein and two had IgG without light chains. All the patients expressing a light chain had a κ paraprotein (Heriot et al. 1985).

In 1987 Crapper *et al* studied 130 homosexual men for the presence of monoclonal bands on serum protein electrophoresis. Sixty-five were HIV positive and 65 negative. The mean age was 33 years (21-42 yrs). Monoclonal bands were found in 6 patients in the HIV positive group and in none of the HIV negative group. Four were single paraprotein peaks and two were multiple oligoclonal bands. The prevalence of monoclonal protein was 9% (Crapper et al. 1987).

In 1987 Lefrere *et al* screened 243 HIV positive patients diagnosed as HIV positive on ELISA for monoclonal proteins. Their mean age was 34 years. Monoclonal proteins

were found in 6 patients (2.5%), all IgG κ and IgG λ. No Bence Jones proteins were present. The $CD4^+$ counts were higher than 200 in the six patients. The peaks of the monoclonal protein varied in size from 2-7 g/L (Lefrere et al. 1993).

In 2007 Konstantinopoulos *et al* investigated 320 HIV-infected patients, of which 253 were males and 67 were females with a mean age of 42 years (7 – 67 years). There was no significant difference in the viral load and the $CD4^+$ T-cell count between males and females. Females had a higher average IgG, but the difference in IgM and IgA was not significant. There were 139 with increased IgG, 72 with increased IgA and 35 with increased IgM levels. In 11 samples, all three immunoglobulins were increased and in 1 sample all three immunoglobulins were decreased. Of the 14 (4.4%) monoclonal bands, 13 were of the IgG κ type and 1 was IgG λ. The average size of the monoclonal peak was 1.85 g/L (range 0.3 - 4.65 g/L). Of the 26 (8.1%) samples with oligoclonal bands, 24 patients had only IgG bands. There were 13 samples with κ and λ bands present and 11 samples had bands with a single light chain type. Two oligoclonal samples had an IgA or IgM band together with an IgG band. The 4 factors associated with banding according to their study were younger age, female sex, increased viral load and $CD4^+$ T-cell count of $\geq$ 350/μL. The study supports the hypothesis that elevated total IgG levels show oligoclonal or monoclonal bands as part of an immune response directed toward HIV and with increased HIV viral load the host B-cells respond by making more immunoglobulins directed at specific HIV epitopes, which can be detected as oligoclonal or monoclonal bands on serum protein electrophoresis. The prevalence of bands in HIV-infected patients in this study were lower than previously reported, perhaps due to more effective ART available (Konstantinopoulos et al. 2007).

In a recent local study by Jansen van Vuuren *et al* published in 2010, 368 HIV- infected patients were investigated. There were 127 males and 241 females enrolled with a mean age of 37.7(17-70) years. The males were older than the females with a mean age of 40 years for males and 36.5 years for females. Monoclonal bands were found in 12 patients (3.2%). Oligoclonal bands were found in 14 patients (3.8%). Three hundred and twenty four patients were on ART for a mean duration of 19.6 months. The presence of bands

was associated with a shorter duration of ART. Viral load and $CD4^+$ count did not differ significantly from patients without monoclonal bands. Most monoclonal bands were of low concentration and of the IgG isotype. IgG was the only heavy chain isotype identified and was present in 10/12 (83%) monoclonal bands and 9/14 (64%) of the oligoclonal bands. Kappa was the light chain most often found in monoclonal bands, 9/12(75%). Oligoclonal bands had 7/14 (50%) κ light chains only and the other 7/14 (50%) had both κ and λ light chains. One patient had only an IgG heavy chain without a light chain. The study confirms that the prevalence of monoclonal and oligoclonal bands are higher in the HIV positive patients on ART compared to the general population (Jansen van Vuuren et al. 2010).

Another South African study was published in 2011, where a group from Kwazulu-Natal performed a retrospective, anonymous analysis of routine laboratory results to describe the effect of HIV status on serum protein electrophoresis patterns. They examined 331 serum protein electrophoresis patterns of routine tests sent to the laboratory. One hundred and two of these were HIV seropositive and 229 HIV seronegative. Those without HIV status results were excluded from the study. They found that monoclonal bands were not increased in HIV-positive patients, but these patients were younger and had a higher incidence of polyclonal and oligoclonal bands and total proteins when compared to HIV-negative patients (Tathiah et al. 2011).

1.5 SIGNIFICANCE OF MONOCLONAL BANDS IN HIV PATIENTS

The significance of these bands in HIV-infected subjects has been investigated in numerous studies.

In 1989 Ng *et al* studied seven HIV–infected subjects with paraproteins in their serum and performed immunoglobulin subclass typing. They found that five had IgG1 κ, one had IgG3 λ and one had IgA λ. They found that IgG1 paraproteins in the sera of HIV-1 infected subjects reflect a vigorous and normal polyclonal immune response to HIV-1

viral antigens and the clinical significance of the IgG3 λ and IgA λ paraprotein was unclear (Ng et al. 1989).

In 1992 Turbat-Herrera *et al* investigated 27 bone marrow aspirates and biopsies of HIV positive subjects with plasmacytosis for the amount of plasma cells. Serum protein electrophoresis and immunoelectrophoresis was performed on 18 of the patients. Five had monoclonal proteins, 11 had polyclonal hypergammaglobulinaemia and 2 had a normal serum protein electrophoresis pattern. The 5 patients with monoclonal paraproteins were identified as two with IgA κ, one with IgG λ and two with IgG κ. There were 5-30% plasma cells in the bone marrow with 1-20% atypical plasma cells. All the subjects were polyclonal on immuno-histochemical staining. There was no correlation between bone marrow plasmacytosis and monoclonal proteins on protein electrophoresis (Turbat-Herrera et al. 1993).

Amara *et al* performed a retrospective analysis on 25 HIV positive patients (24 males and 1 female) with detectable serum monoclonal protein in 2006. The mean age was 44 years (21-69 years). Various clinical presentations led to the finding of monoclonal proteins with serum protein electrophoresis. Serum monoclonal proteins varied between 2-60g/L with a mean monoclonal concentration of 30g/L. Of the 25 monoclonal proteins, 24 were of the IgG type and 1 IgA. Urine protein immunoelectrophoresis was performed on 20 of the 25 patients with Bence Jones. Serum immunoglobulin was performed on 23 patients. Seven had normal immunoglobulin, 14 had IgG hypergammaglobulinaemia and 2 had hypogammaglobulinaemia. There was no correlation with the $CD4^+$ count. After a follow-up duration of 21 months, 9 of the 16 monoclonal peaks decreased, 7 did not decrease and none disappeared while on HAART. Seven (28%) of the 25 patients developed a malignancy. One patient developed MM, 1 marginal zone lymphoma, 2 Kaposi sarcoma, 2 plasmacytoma and 1 testicular cancer, suggesting an increased risk for malignancy in these patients (Amara et al. 2006).

In 1992 Lefrere *et al* performed a study on 341 symptomless HIV positive subjects diagnosed on ELISA over a 6 year period to detect serum monoclonal proteins. Eleven

of the 341 subjects had monoclonal proteins. Of the 11 subjects, 7 monoclonal peaks disappeared with follow-up, 2 individuals developed a second peak and 1 individual a third peak. They concluded that monoclonal proteins in symptomless HIV-infected subjects does not signify a prelymphomatous state and may not be used as a predictive marker of disease progression (Lefrere et al. 1993).

Pontet *et al* studied the immunoglobulins of 212 HIV-positive patients and followed them up for 13 years (1984-1997). The qualitative features of the immunoglobulin can be divided into 3 groups, monoclonal Ig, minor abnormalities and polyclonal immunoglobulin. The incidence of monoclonal immunoglobulin was 11.3%. The (male/female) sex ratio was 2.2 with 12.1% and 11.0% of monoclonal Ig in the female to male respectively. The male/female ratio for monoclonal Ig is 0.91. There was no increase in monoclonal immunoglobulin with age in female or in males. Minor abnormalities were more in females (29%) than in males (18%). This study showed that prevalence of monoclonal immunoglobulin is higher in HIV-positive than in the general population of the same age. The survival curve shows that the presence of immunoglobulin abnormalities, monoclonal or minor, in HIV-positive patients has no prognostic significance (Pontet et al. 1998).

1.6 FREE LIGHT CHAINS

In 1962 Edelman and Gally showed that free light chains (FLC) prepared from IgG monoclonal proteins were the same as Bence Jones protein (Edelman and Gally. 1962). Antibody molecules have a two-fold symmetry and are composed of two identical heavy chains and two identical light chains, each containing variable and constant domains. The variable domains of each light chain, heavy chain pair combines to form an antigen-binding site so that both chains contribute to the antigen-binding specificity of the antibody molecule as shown in figure 1.2. Light chains are of two types, kappa (κ) and lambda (λ) and any given antibody molecule has either κ or λ chains, but never both. There are twice as many κ as λ molecules produced in humans (Edelman and Gally. 1962).

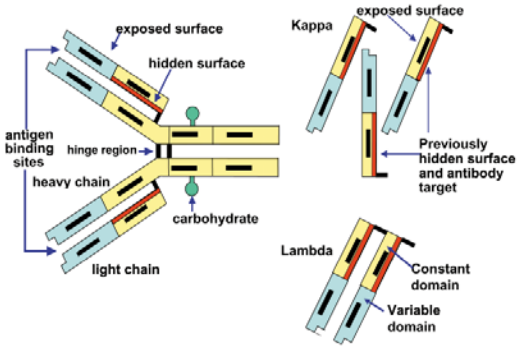

Figure 1.2 The antibody molecule showing the heavy and light chain structure

Normal and abnormal plasma cells produce more light chains than heavy chains and the excess light chains are released into the bloodstream. The FLC's are cleared and metabolized by the kidneys. Increases in serum FLC levels may occur due to decreased renal clearance. In patients with impaired renal clearance, the serum light chain levels may be elevated, but the free light chain κ:λ ratio will be normal. Patients with an expansion of either κ or λ producing plasma cell clone, have an abnormal serum FLC κ:λ ratio. This ratio is a very sensitive diagnostic test for plasma cell clones that have lost the ability to produce heavy chains and secrete only light chains. Kappa monomeric FLC's are smaller in size (25 kDa) than the λ dimeric FLC's (50 kDa); therefore the κ monomeric molecules filter approximately three times faster (Solomon. 1985; Waldman et al. 1972; Miettinen and Kekki. 1967; Arfors et al. 1979).

Immunoassays based on polyclonal antibodies were developed that could measure FLC's at normal serum concentrations (Bradwell et al 2001). Their utility was made apparent when monoclonal FLC's were detected in serum of patients classified as having "nonsecretory" myeloma (Drayson et al. 2001). FLC determination is important in the diagnosis of monoclonal light chain diseases such as primary amyloid and disorders that often do not have serum monoclonal proteins in high enough concentration to be detected and quantitated by protein electrophoresis (Katzmann et al. 2005).

The serum FLC analysis provides a risk assessment for the progression of MGUS to MM. In a study where baseline serum samples were obtained with 30 days of diagnosis of

MGUS in 1148 patients, 737 (64%) had elevated levels of κ and λ FLCs. An abnormal FLC ratio was detected in 379 (33%) patients. The risk of progression to MM or related malignancy at 10 years was 17% with an abnormal ratio compared with 5% with a normal ratio (Rajkumar et al. 2005). If MGUS is diagnosed, the FLC ratio can be used to segregate patients into high and low risk groups. Patients with an abnormal FLC ratio have a 2.5 fold increased risk of progression (Rajkumar et al. 2005; Kyle et al. 2006). The serum FLC assay can be used to monitor the disease course in patients with a monoclonal protein that cannot be measured by protein electrophoresis. By using the assay to monitor MM patients who present with unmeasurable levels of monoclonal protein in serum or urine protein electrophoresis, physicians can minimize the use of serial bone marrow biopsies and reduce the use of invasive techniques. Furthermore, by measuring the serum FLC in a patient's serum, the need for collecting a 24-hour urine sample is eliminated. This is advantageous, as urine collection is inconvenient for patients and the laboratory workload is reduced by eliminating the need to process urine samples (Rajkumar et al. 2005).

In a study of 273 patients with smouldering MM, the light chain type was κ in 68% and λ in 32%. An abnormal FLC ratio was detected in 245 patients (90%). An increasingly abnormal FLC ratio was associated with a higher risk for progression to active MM. Patients with a normal or near normal ratio had a rate of progression of 5% a year, while patients with increasingly abnormal ratio had a progressive increase in the risk of progression of about 8.1% per year (Rajkumar et al. 2004).

In a study of 116 patients who met the criteria for solitary bone plasmacytoma, 53% had a normal FLC ratio and 47% had an abnormal ratio. Patients with an abnormal ratio had a higher incidence of monoclonal protein in the urine and a larger serum monoclonal spike (Dispenzieri et al. 2008).

Bradwell *et el* and Nowrousian *et al* showed that patients with LCMM have abnormal concentrations of serum FLC and abnormal κ/λ ratios and that immunoassays for serum FLC are more sensitive for identifying of Bence Jones protein than immunofixation of

urine (Bradwell et al. 2003; Dingle et al. 2006). Drayson *et al* showed that patients with nonsecretory MM have elevated κ or λ FLC concentration and abnormal κ/λ ratios (Drayson et al. 2001). In study by Mead *et al* it was found that patients had elevated, normal or reduced concentration of FLC but the κ/λ ratios were all abnormal (Nowrousian et al. 2003).

Patients with Waldenströms macroglobulinaemia have abnormal FLC concentrations and/ or κ/λ ratios (Mead et al. 2004). Various studies showed that serum FLC measurement is a useful screening test and supplement other tests and the quantitative nature of FLC immunoassays have a value in monitoring patients (Bradwell et al. 2002; Lachmann et al. 2003; Katzmann et al. 2002; Abrahams et al. 2003).

1.6.1 Reference intervals

Reference intervals for FLC's were established at the Mayo Clinic using 282 mainly Caucasian elderly subjects (Katzmann et al. 2002). No local reference intervals have been established in South Africa yet, although it has been advised to establish local reference intervals (Pattenden et al. 2007).

Racial differences have been described in the prevalence of MGUS and MM (Weiss et al. 2011; Landgren and Weiss. 2009) and in immunoglobulin levels in HIV subjects (McGowan et al. 2006). As most of our cohort was younger subjects with HIV of either black or mixed ancestry ethnicity, the need to verify the manufacturer's reference intervals was identified.

1.6.2 FLC Assay:

Laboratory investigations for possible B-cell disorders currently require both urine and serum samples (Akar et al. 2005). B-cell disorders cannot be excluded without analysis of urine samples by existing methods, because LCMM and NSMM account for 15% - 20% of new diagnoses. In many of these cases the serum FLC concentrations are below the detection limits of conventional immunofixation methods (Akar et al. 2005). It is often

difficult to collect serum and urine samples and concurrent urine samples are received from less than 40% of patients (Akar et al. 2005).

The serum FLC automated assay was developed in the early 2000s to detect light-chain epitopes that are exposed *only* when not bound to a heavy chain. The assay quantifies κ and λ FLC's and is now routinely used in the diagnosis and management of several plasma cell proliferative disorders, including MGUS, light-chain amyloidosis and MM (Hill et al. 2006).

Studies have shown that serum FLC assays are more sensitive than serum protein electrophoresis and urine protein electrophoresis for the detection of urine light chains in MM, NSMM and primary amyloidosis. Serum FLC cannot replace serum protein electrophoresis in a screening protocol for monoclonal gammopathies, because they are slightly less sensitive when screening for intact immunoglobulin MM. However, they have the potential to replace urine protein electrophoresis, as they have a lower limit of detection for FLC and a high percentage of serum samples are sent without concurrent urine samples. Serum FLC in addition to serum protein electrophoresis may improve the detection of monoclonal gammopathies (Akar et al. 2005).

A recent study examining only serum samples showed that additional patients with B-cell disorders were identified when serum FLC measurement was used in conjunction with capillary zone electrophoresis (Bakshi et al. 2005).

However, the FLC assay is not without its limitations. Variation in assay reagents in different lot numbers can be a problem. Both polyclonal and monoclonal serum FLC's have been found to dilute in a nonlinear fashion, leading to underestimation in the absence of off-line dilution (Bakshi et al. 2005). False high results may occur due to polymerization of light chains (Tate et al 2007; Abrahams et al. 2002). In a study by Hill *et al* false positive results were detected (Hill et al. 2006). Increased immunoglobulins, renal impairment and/or acute phase responses lead to more false positive results (Akar et al. 2005). False low serum FLC results may be obtained due to antigen excess when very

high concentrations are present (Édmond et al. 2007). Nephelometric assays measure light scatter caused by the formation of immune complexes in solution and are subject to limitations inherent in antigen-antibody reactions. Newly identified cases with abnormally high serum FLC ratios should be retested with a higher dilution, because of the potential for FLC antigen excess (Hill et al. 2006). It has been proposed that serum protein electrophoresis and serum FLC's be used as first line tests for the investigation of possible B-cell disorders, because no substantial pathology would have been missed by replacing urine Bence Jones Protein with serum FLC's (Akar et al. 2005). However, the assay has also been criticized for its high level of imprecision, the variations on various platforms and the fact that assays and standards are only provided by one company (Sheldon 2007).

Method validation is a standard process in every good laboratory in judging the acceptability of a new method (Percy-Robb et al. 1980). The decision on acceptability depends on defining quality standards that provide objective statements on how good a test should be. These quality standards "criteria for acceptable performance" can be retrieved from sources such as CLIA or be based on biological variation data.

Dr Westgard refers to method validation simply as "*error assessment*". The focus is on analytical errors and how these errors impact in the interpretation of a test. While a comparison of method experiment can reveal the different type of errors, there are specifically designed experiments for each one of them (Westgard 2008). The different type of errors observed and the experiments required in identifying them are shown in table 1.1.

Table 1.1 Method validation studies and the errors they detect

Type of Analytical Error	**Evaluation Experiment**	
	Preliminary	Final
Random Error	Replication	
Constant Error	Interference	Comparison of Methods
Proportional Error	Recovery	
	Linearity experiment	

1.7 PRESENT KNOWLEDGE OF FLC CONCENTRATION IN HIV

A Pubmed search showed that there is currently only one study examining FLC in HIV infection. Landgren *et al* found that FLC were elevated in HIV-infected subjects compared to the general population, and strongly predicted NHL risk, independent of $CD4^+$ count. In contrast, markers of monoclonal B-cell proliferation (abnormal FLC ratio) were not associated with NHL development. FLC's may be a sensitive marker of polyclonal B-cell activation or dysfunction and could identify HIV-infected person at increased NHL risk (Landgren et al. 2010). De Filippi *et al* reported on 2 HIV-negative subjects presenting with primary effusion lymphoma (PEL). These patients present with *HIV-like* symptoms (De Filippi et al. 2009).

A recent publication in Clinical Chemistry (Hutchison and Landgren. 2011) discussed the use of FLC measurement as a marker of immune stimulation and inflammation. They discussed its potential use as a biomarker of activation of the B-cell lineage and mentioned the study by Landgren *et al* (Landgren et al. 2010).

1.8 HYPOTHESIS

FLC is a marker of B cell dysfunction, and as HIV is associated with B cell dysfunction, we hypothesized that FLC levels and FLC ratio will be abnormal in HIV-infected individuals.

1.9 AIMS OF STUDY

1) To validate the FLC Assay using the Beckman Immage®
2) To develop local FLC reference intervals
3) To determine FLC levels and κ/λ ratios in HIV positive subjects (mainly on HAART)

SECTION II: MATERIALS AND METHODS

2.1 METHOD VALIDATION:

As described in the literature review, method validation is performed on all new tests prior to their routine introduction in the laboratory as a means of evaluating errors.

2.1.1 Linearity study

The linearity study was performed in 2 parts, namely on linearity fluid and on pooled serum samples obtained from routine samples sent to the Chemical Pathology laboratory. The linear range specified by the manufacturer is 6.0-180mg/L for κ FLC and 4.8-162 mg/L for λ.

2.1.1.1 Using Linearity Fluid

The κ fluid was diluted to a value of 64.47 mg/l, which is in the linear range, using dilutions made according to the Freelite™ instructions. These were then analysed on the Beckman IMMAGE® in triplicate to negate the effect of random errors (imprecision) and the results were then compared to the assigned values of the linearity fluid.

The λ fluid was diluted to a value of 135.67 mg/l, which is in the linear range, using dilutions made according to the Freelite™ instructions. These were then analysed on the Beckman IMMAGE® in triplicate to negate the effect of random errors (imprecision) and the results were then compared to the assigned values of the linearity fluid.

2.1.1.2 Using Pooled Serum

Serum obtained from routine samples sent to the Chemical Pathology laboratory was pooled and a κ value of 38.92 mg/l was obtained, which is in the linear range. Dilutions were made according to the Freelite™ instructions using the manufacturer's diluent. These were then analysed on the Beckman IMMAGE® in triplicate to negate the effect of

random errors (imprecision) and the results were then compared to the determined values of the pool sample.

Serum obtained from routine samples sent to the Chemical Pathology laboratory was pooled and a λ value of 49.47 mg/l was obtained, which is in the linear range. Dilutions were made according to the Freelite™ instructions using the manufacturer's diluent. These were then analysed on the Beckman IMMAGE® in triplicate to negate the effect of random errors (imprecision) and the results were then compared to the determined values of the pool sample.

2.1.2 Recovery experiment

2.1.2.1 Kappa Recovery:

Six patient samples with different concentrations of κ FLC were obtained and spiked with a high κ control (31.9 mg/l). This control was spiked into an aliquot of the patient samples in a 1:10 dilution. This sample was then analyzed in duplicate to negate the effect of random errors and the recovery was then calculated for each individual sample.

2.1.2.2 Lambda Recovery:

Five patient samples with different concentrations of λ FLC were obtained and spiked with a high λ control (46.2 mg/l). This control was spiked into an aliquot of the patient samples in a 1:10 dilution. This sample was then analyzed in duplicate to negate the effect of random errors and the recovery was then calculated for each individual sample.

2.1.3 Interference Studies

2.1.3.1 Haemolysis interference study

A haemolysate was prepared using the modified osmotic shock method in the laboratory and samples with different haemoglobin (Hb) concentrations were made using the above haemolysate. The final Hb concentrations were as follows: 12.1 g/dl, 4.15 g/dl, 3.25 g/dl, 2.5 g/dl and 2.1g/dl. Four serum pools were made, namely high κ (50.8 mg/l), high λ (48.8 mg/l), low κ (10.85 mg/l) and low λ (19.15 mg/l). The interferent (Hb) was then spiked into the above pools in a dilution of 1:10. These samples were analyzed in duplicate to negate the effects of random errors. A ± 10% allowable error from baseline was used to determine acceptability for the different Hb concentrations as advised by the manufacturer.

2.1.3.2 Bilirubin interference study

A sample with a bilirubin of 371.2 µmol/l was used in the interference study. Samples with different bilirubin concentrations were made by diluting the above sample. The final bilirubin concentrations were as follows: 371.2 µmol/l (neat), 117.4 µmol/l, 86.7 µmol/l, 71.3 µmol/l and 41.7 µmol/l. Four serum pools were made, namely high κ (33.45 mg/l), high λ (39.65 mg/l), low κ (16.7 mg/l) and low λ (18.1 mg/l). The interferent (bilirubin) was spiked into the above pools in a dilution of 1:10. These samples were then analyzed in duplicate to negate the effects of random errors. A ± 10% and ± 20% allowable error from baseline was used to determine the acceptability for the different bilirubin concentrations as advised by the manufacturer.

2.1.3.3 Rheumatoid factor interference study

A sample with a rheumatoid factor (RF) value of 1850 IU/ml was retrieved from the department of Immunology for the interference study. The sample was diluted using the Beckman diluent, creating concentrations of 581, 486, 385 and 311 IU/ml. Four serum

pools were made, namely high κ (45.00 mg/l), high λ (50.65 mg/l), low κ (10.17 mg/l) and low λ (20.8 mg/l). The interferent (RF) was spiked into the above pool in a dilution of 1:10. These samples were then analyzed in duplicate to negate the effects of random errors. A ± 10% and ± 20% allowable error from baseline was used to determine the acceptability for the different RF concentrations as advised by the manufacturer.

2.1.3.4 Triglyceride interference study

A lipaemic sample with a triglyceride value of 25.82 mmol/l was used for the interference study. Samples with different triglyceride concentrations were made by diluting the above sample. The final triglyceride concentrations were as follows: 25.82 mmol/l (neat), 9.31 mmol/l, 7.74 mmol/l, 5.79 mmol/l and 5.06 mmol/l. Four serum pools were made, namely high κ (45.00 mg/l), high λ (50.65 mg/l), low κ (10.17 mg/l) and low λ (20.8 mg/l). The interferent (triglyceride) was spiked into the above pools in a dilution of 1:10. These samples were then analyzed in duplicate to negate the effects of random errors. A ± 10% and ± 20% allowable error from baseline was used to determine the acceptability for the different triglyceride concentrations as advised by the manufacturer.

2.1.4 Imprecision Evaluation

We utilized the CLSI EP15 protocol to verify the performance for precision and trueness of the test (Chesher 2008).

Precision was tested using pooled serum samples (high and low pool) for both κ and λ light chains. Trueness was verified by using control material (high and low controls) for both κ and λ.

2.1.5 Method Comparison Study

A comparison of methods experiment is performed to estimate the bias i.e. the inaccuracy or systematic error of a method. This is carried out by analyzing patient samples on the

new method and on the comparative method. Forty samples were used to compare κ FLC and 38 to compare λ FLC. These samples were collected over a time period of one month. As they could not be analysed within two hours of each other, the samples were stored at -70°C. The systematic errors are based on the differences observed between the methods. It is therefore important that the statistics calculated provide information about the systematic error at medically important decision levels. The important statistics include the correlation coefficient, regression equation and the difference plot.

Since the FLC test has not been introduced on the regular test menu of our laboratory, we compared our method (nephelometric on the Beckman Immage®) to a same established method used at another laboratory.

2.1.5.1 Correlation

Correlation describes the level of agreement between two methods and is a very important statistical tool that confers reliability of the regression statistics calculated.

2.1.5.2 Regression statistics

The relevant errors in a method evaluation include the random, proportional and constant errors. The regression statistics is an important statistical tool that allows one to estimate the above errors. The systematic error can also very importantly be estimated at any concentration using the regression equation. This is important in assessing the systematic error at key medical decision limits.

In view of the limitations observed with simple linear regression statistics, Deming's Regression Statistics was used in the analysis.

2.1.5.3 Difference plot (Bland-Altman)

The difference or Bland-Altman plot allows for a graphical display of the bias observed in relation to the comparative method. It is constructed as follows: The mean of x_i and y_i is determined for every sample pair .Y_i is then subtracted from the calculated mean and expressed graphically. This plot allows for a more objective appreciation of the bias at key decision limits.

2.2 ESTABLISHING LOCAL SERUM REFERENCE INTERVALS:

Blood was obtained from 120 normal subjects, namely 78 healthy HIV negative blood donors and 42 healthy black volunteers who were participating as controls in another study being performed by the Division of Haematology. The following blood tests were performed on each subject: total protein, IgG, IgA, IgM, creatinine, protein electrophoresis, κ FLC and λ FLC. Using the results of κ and λ FLC, a FLC ratio was determined. Informed consent was obtained from all subjects prior to enrolment (see Appendix 1) and participation was voluntary.

2.3 FLC IN HIV PATIENTS

This was a retrospective descriptive study, using blood samples stored at -70°C from a previous study obtained from patients with HIV infection attending clinics at Karl Bremer and TC Newman Hospitals in the Western Cape. Stability studies on FLC's are still ongoing, but after discussion with the scientific advisor of the Binding Site, it was decided that the samples would be stable at this temperature for this time period.

2.3.1 Inclusion Criteria

Any patient, known with HIV infection, whether receiving ART or not, followed up at Karl Bremer or TC Newman Infectious Diseases Clinic was eligible for enrolment in the study. Patients were enrolled consecutively as they presented for follow-up at the clinic.

2.3.2 Exclusion Criteria

Children were excluded from this study.

2.3.3 Clinical Data

Clinical information was recorded by means of a form filled in by the investigator from information in the medical record and by means of patient interview. The data collected included:

- Demographic details: age, gender and ethnic group.
- Stage of disease: WHO stage of disease, most recent $CD4^+$ count and viral load.
- Medical history: previous and current serious illness e.g. tuberculosis, malignancy, auto-immune disease or viral hepatitis.
- ART: duration of treatment.

2.3.4 Ethical Consideration

The study was carried out in accordance with the Declaration of Helsinki and ICH GCP guidelines. The study protocol was approved by the University of Stellenbosch Ethics Committee (see Appendix 2). All patients signed an informed consent form (see Appendix 3). Patient confidentiality was maintained at all times. The data capture sheets containing clinical and demographic information reflected a study number only, and specimens and results were labeled with the study number only. Identifying information was kept separately to protect confidentiality.

2.3.5 Determination of FLC

Kappa and λ FLC were determined on the Beckmann Coulter IMMAGE® by nephelometry. It involves the addition of the serum sample to a solution containing the appropriate antibody in a cuvette. A beam of light is passed through the cuvette and as the antigen-antibody reaction proceeds, the light passing through the cuvette is scattered

increasingly as insoluble immune complexes are formed. The scattered light is monitored by measuring the decrease in intensity of the beam of light. The antibody in the cuvette is in excess, thus the amount of immune complex formed is proportional to the antigen concentration.

2.3.6 Determination of Total Protein

Total protein used for quantifying the various protein fractions by densitometry, was determined on the ADVIA™ 1800 (Siemens) clinical chemistry analyzer. The method is based on the method of Weichselbaum, utilizing the biuret reagent and measuring the endpoint reaction at 545nm. Total protein is reported in gram per litre (g/L).

2.3.7 Determination of Immunoglobulins

IgG, IgA and IgM were determined by polyethylene glycol-enhanced immunoturbidimetric method on the ADVIA™ 1800 (Siemens). Polyethylene glycol accelerates the antigen-antibody interaction as described in the work of Hellsing.

2.3.8 Determination of Creatinine

Creatinine was determined on the Beckman Coulter CX7® by means of the Jaffe rate method. Creatinine is reported in µmol/l.

2.3.9 Determination of albumin and gamma globulins

Albumin and gamma globulins were determined densitometrically from the electrophoresis using agarose gel electrophoresis.

2.3.10 Data Processing

Data was analyzed with the help of a statistician, using STATISTICA version 10 (StatSoft Inc.) and Microsoft® Excel®. The calculations were performed in STATISTICA and Microsoft® Excel® was used to graphically display the results of the calculations.

Descriptive statistics were used to analyze each parameter in terms of distribution, mean, median, quartiles, maximum and minimum values and standard deviation.

Continuous variables were compared against each other using regression and correlation analysis. For data with a normal distribution, Pearson's correlation coefficient was performed. The Spearman rank correlation coefficient was used for data with a non-normal distribution (Pipkin).

The Analysis of Variance (ANOVA) was used for non-continuous variables to compare two or more groups if the data are normally distributed.

SECTION III: RESULTS

3.1 METHOD VALIDATION

3.1.1 Linearity study

3.1.1.1 Using Linearity Fluid

The results were linear within the recommended range for both κ and γ FLC's using linearity fluid, as shown in figures 3.1 and 3.2.

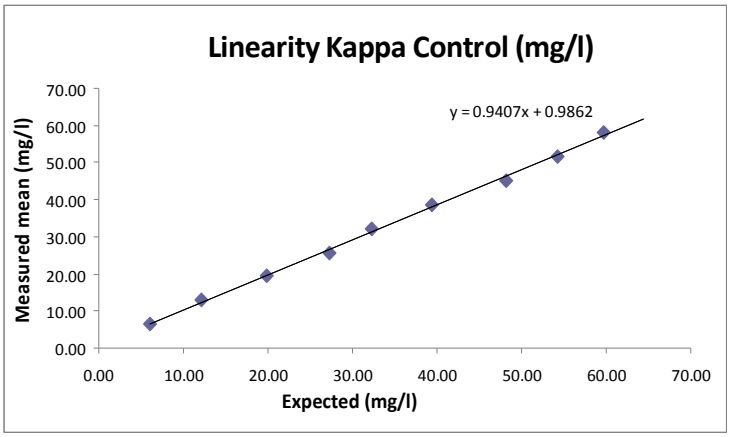

Figure 3.1 Results of the linearity study for κ FLC using linearity fluid provided by the manufacturer

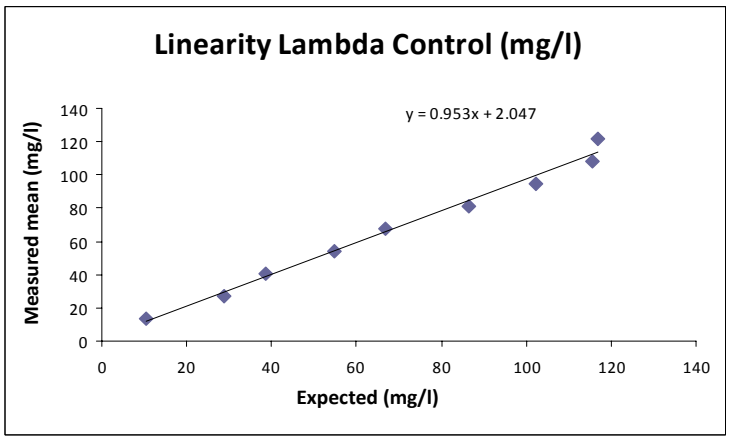

Figure 3.2 Results of the linearity study for λ FLC using linearity fluid provided by the manufacturer

3.1.1.2 Using Pooled Serum

The results were linear within the recommended range for both κ and λ FLC's using pooled serum samples as shown in figures 3.3 and 3.4.

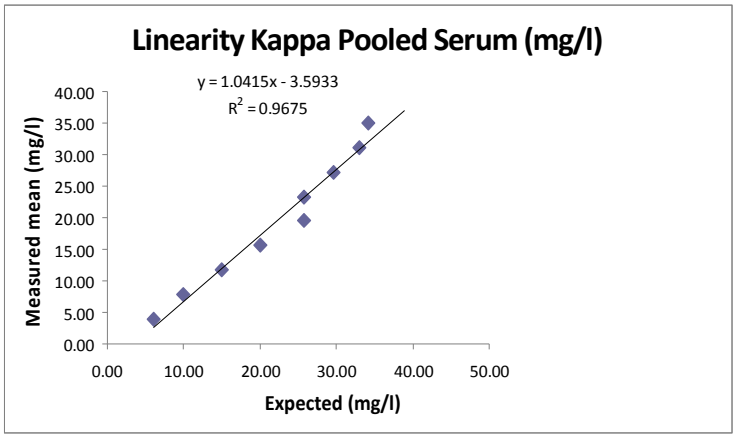

Figure 3.3 Results of the linearity study for κ FLC using pooled serum

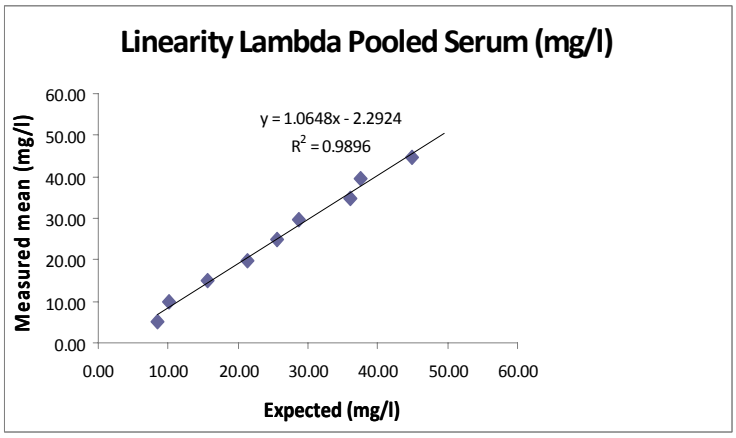

Figure 3.4 Results of the linearity study for λ FLC using pooled serum

3.1.2 Recovery experiment

3.1.2.1 Kappa Recovery:

κ Control (high) 31.9 mg/l

	Base	Spiked	Difference	Added	% Recovery
Sample 1	10.90	13.00	2.10	3.19	65.80
Sample 2	4.60	7.23	2.63	3.19	82.45
Sample 3	13.90	14.35	0.45	3.19	14.00
Sample 4	23.75	24.10	0.35	3.19	10.97
Sample 5	19.35	20.40	1.05	3.19	32.91
Sample 6	12.20	13.35	1.15	3.19	36.05
AVERAGE					**40.36**

The average recovery was calculated to be 40.36%, indicating a large proportional error. This result could be due to matrix effect of individual samples as demonstrated above. After discussion with the manufacturer, it was decided to not repeat this study.
Samples 1 and 2 showed a reasonable recovery, but the rest not.

3.1.2.2 Lambda Recovery

λ Control (high) 46.2 mg/l

	Base	Spiked	Difference	Added	% Recovery
Sample 1	20.15	24.50	4.35	4.62	93.50
Sample 2	42.35	46.80	4.45	4.62	96.32
Sample 3	7.30	11.10	3.80	4.62	82.25
Sample 4	2.47	7.10	4.63	4.62	100.20
Sample 5	10.55	14.50	3.95	4.62	85.50
AVERAGE					**91.55**

The average recovery was calculated to be 91.55%, indicating a proportional error of 8.45%

3.1.3 Interference studies

3.1.3.1 Haemolysis interference study

For κ FLC, the low pool was within the 10% allowable error as shown in figure 3.5. However, the high pool was outside the allowable 10% error at a Hb concentration of 3.23 g/dl as shown in figure 3.6. This could have been due to random error.

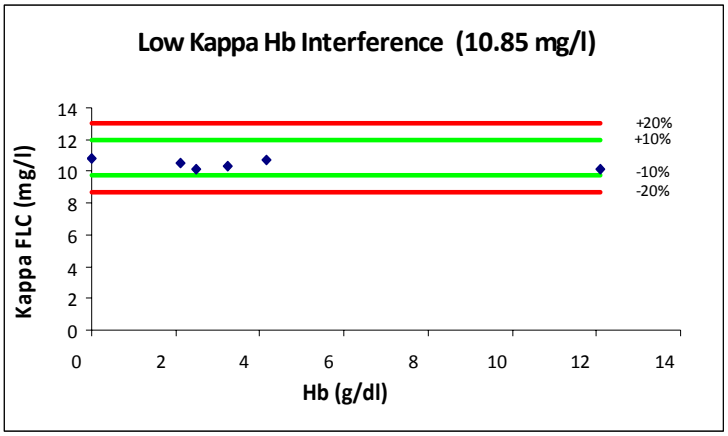

Figure 3.5 Hb interference at low κ FLC levels

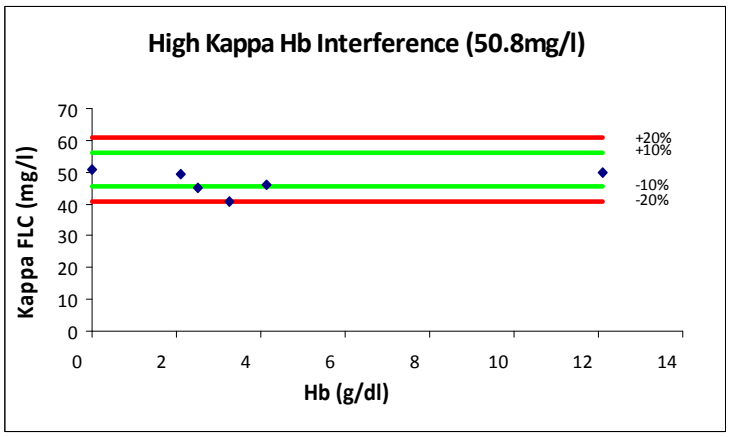

Figure 3.6 Hb interference at high κ FLC levels

As can be seen in figures 3.7 and 3.8, both the low and high pools of λ, the results obtained were within the 10% allowable error indicating minimal Hb interference.

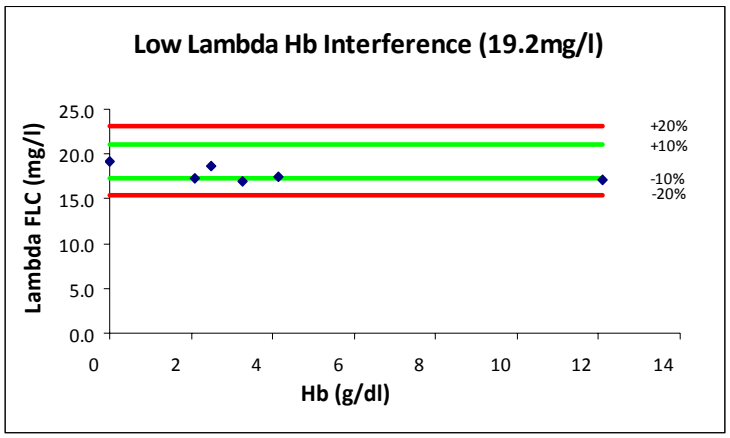

Figure 3.7 Hb interference at low λ FLC levels

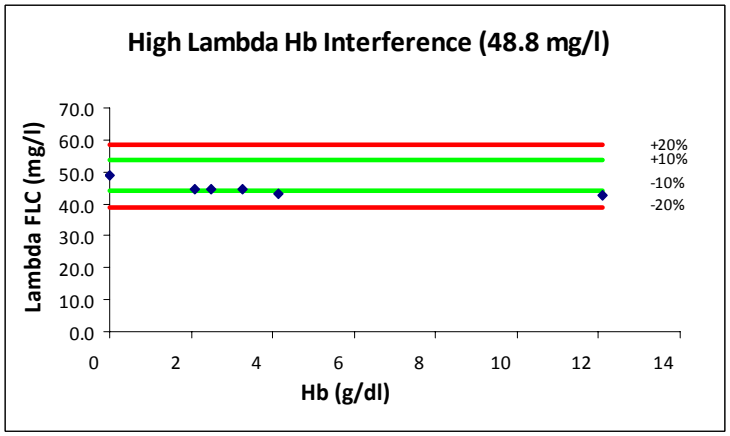

Figure 3.8 Hb interference at high λ FLC levels

3.1.3.2 Bilirubin interference study

Bilirubin did not lead to interference in excess of the allowable 20% deviation with either high or low κ levels, as is shown in figures 3.9 and 3.10.

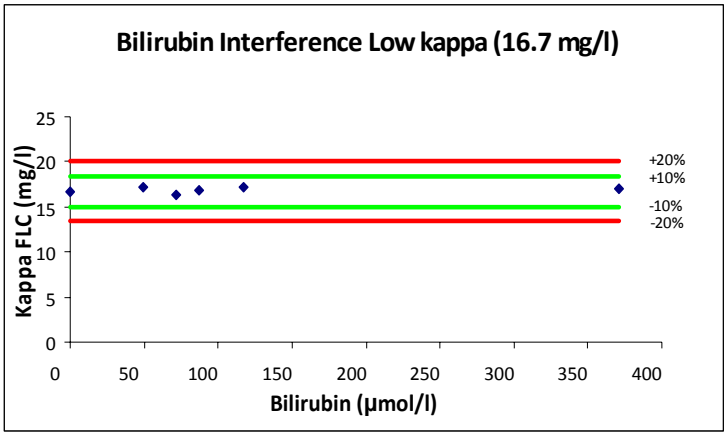

Figure 3.9 Bilirubin interference at low κ FLC levels

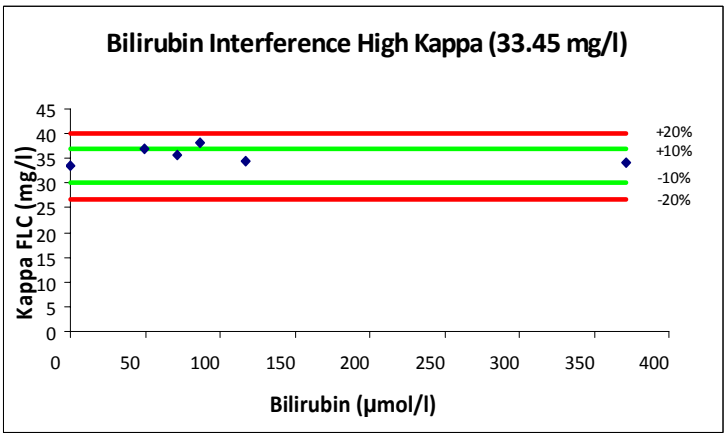

Figure 3.10 Bilirubin interference at high κ FLC levels

Bilirubin did not lead to interference in excess of the allowable 20% deviation with either high or low λ levels, as is shown in figures 3.11 and 3.12.

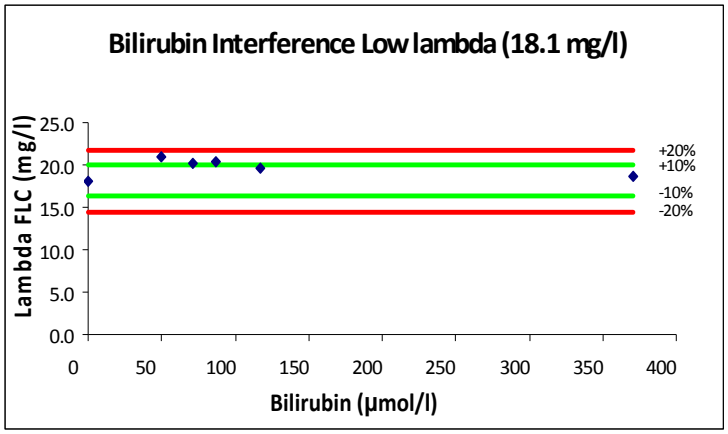

Figure 3.11 Bilirubin interference at low λ FLC levels

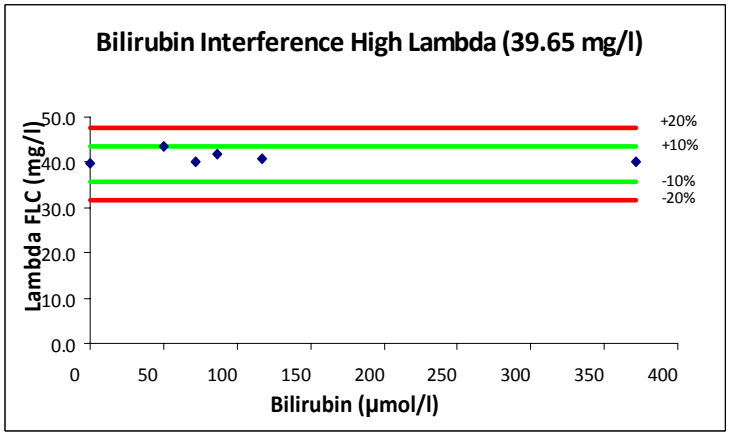

Figure 3.12 Bilirubin interference at high λ FLC levels

3.1.3.3 Rheumatoid factor interference study

RF did not lead to interference in excess of the allowable 20% deviation with either high or low κ levels, as is shown in figures 3.13 and 3.14.

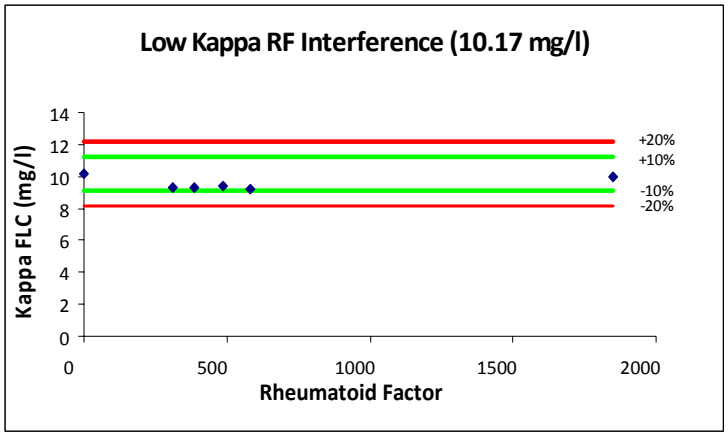

Figure 3.13 RF interference at low κ FLC levels

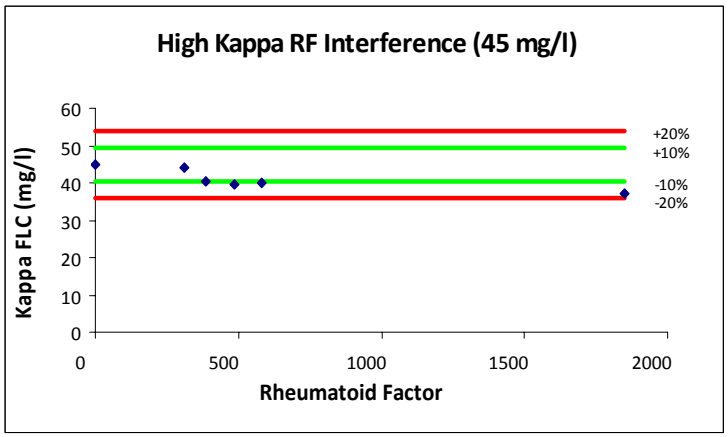

Figure 3.14 RF interference at high κ FLC levels

RF did not lead to interference in excess of the allowable 20% deviation with either high or low λ levels, as is shown in figures 3.15 and 3.16.

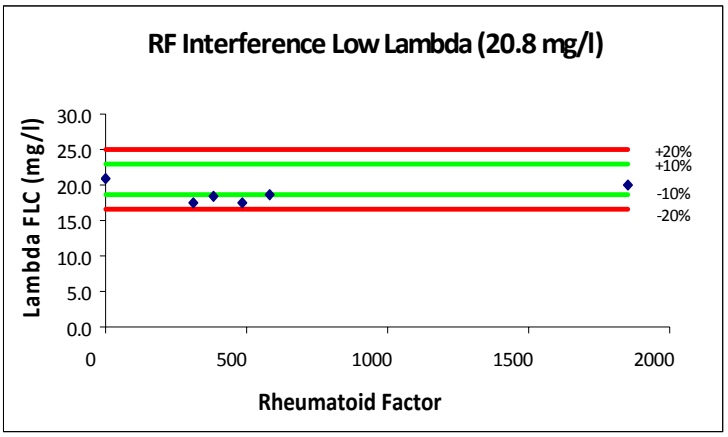

Figure 3.15 RF interference at low λ FLC levels

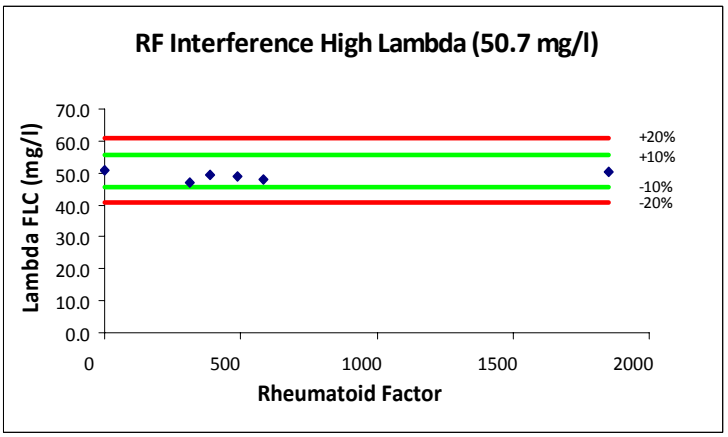

Figure 3.16 RF interference at high λ FLC levels

3.1.3.4 Triglyceride interference study

For κ FLC, no values exceeded the 20% deviation and it was concluded that triglyceride does not interfere with FLC analysis on the Beckman IMMAGE® as shown in figures 3.17 and 3.18.

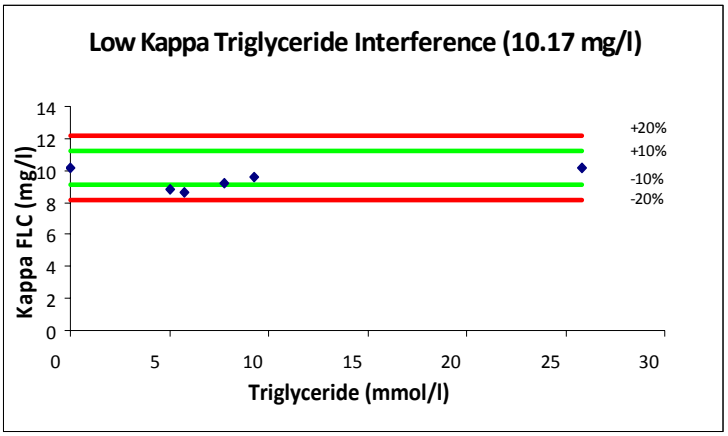

Figure 3.17 Triglyceride interference at low κ FLC levels

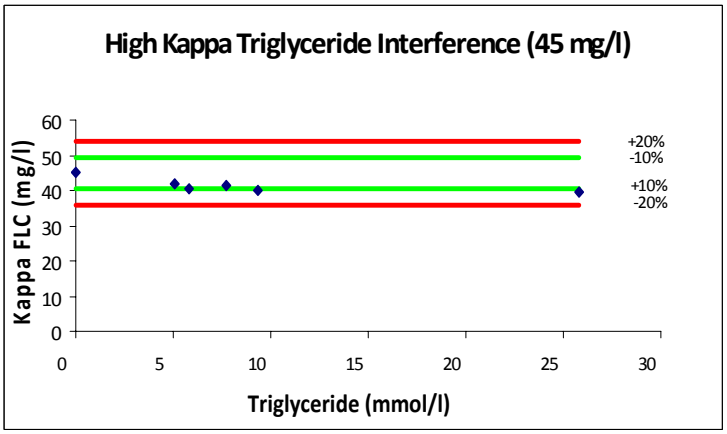

Figure 3.18 Triglyceride interference at high κ FLC levels

Similarly, for λ FLC, no values exceeded the 20% deviation and it was concluded that triglyceride does not interfere with FLC analysis on the Beckman IMMAGE® as shown in figures 3.19 and 3.20.

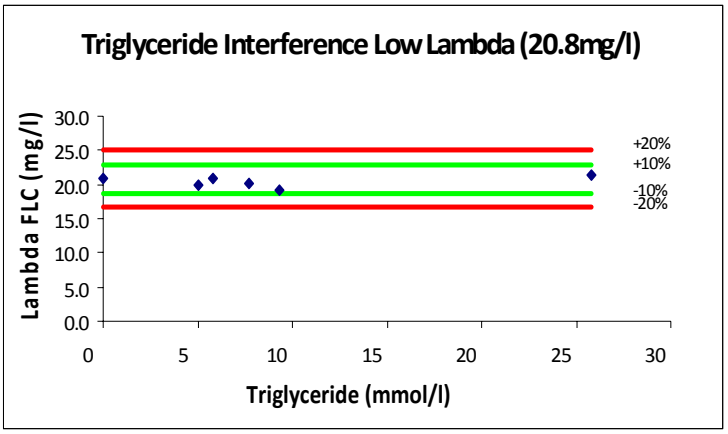

Figure 3.19 Triglyceride interference at low λ FLC levels

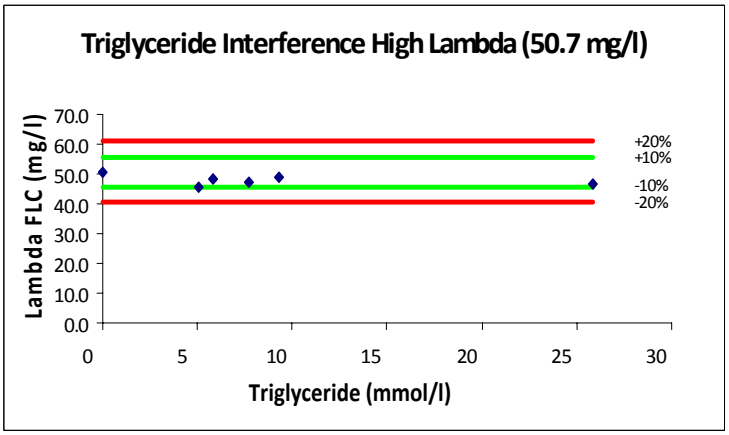

Figure 3.20 Triglyceride interference at high λ FLC levels

3.1.4 Imprecision evaluation

3.1.4.1 Kappa FLC

Using high κ control, trueness was demonstrated, as the assigned value of 32.1 mg/l is included in the 95% confidence interval of 30.36 -33.52mg/l.

Using low κ control, Trueness was demonstrated, as the assigned value of 16.00 mg/l is included in the 95% confidence interval of 15.87 - 17.25mg/l.

The EP15 protocol was used in determining the imprecision of κ FLC (5-day protocol) on a high and low serum pool as shown in Table 3.1 and 3.2

Table 3.1 Results of the imprecision studies for high κ serum pool

High κ Serum Pool (Mean = 33.40g/l)	***SD (mg/l)***	***CV (%)***
Within-run /Repeatability	2.167	6.49
Within-Lab	2.415	7.23

Table 3.2 Results of the imprecision studies for low κ serum pool

Low κ Serum Pool (Mean = 10.84g/l)	***SD (mg/l)***	***CV (%)***
Within-run /Repeatability	0.767	7.08
Within-Lab	0.748	6.9

The precision of the κ FLC assay was acceptable. CV's of <10% observed for all serum pools and trueness of the control material assigned values verified.

3.1.4.2 Lambda FLC

Using high λ control, trueness was demonstrated, as the assigned value of 56.6 mg/l is included in the 95% confidence interval of 50.84 – 57.52 mg/l.

Using low λ control, trueness could not be demonstrated as the assigned value of 28.3 mg/l is not included in the 95% confidence interval of 24.22 -27.56mg/l, but when combined with the experimental uncertainty, trueness was demonstrated by the verification limits of 19.28 - 32.50 mg/l.

The EP15 protocol was used in determining the imprecision of λ FLC (5-day protocol) on a high and low serum pool as shown in Tables 3.3 and 3.4.

Table 3.3 Results of the imprecision studies for high λ serum pool

High λ Serum Pool (Mean = 49.30g/l)	***SD (mg/l)***	***CV (%)***
Within-run /Repeatability	1.907	3.87
Within-Lab	2.088	4.24

Table 3.4 Results of the imprecision studies for low λ serum pool

Low λ Serum Pool (Mean = 19.44g/l)	***SD (mg/l)***	***CV (%)***
Within-run /Repeatability	1.144	5.89
Within-Lab	1.140	5.86

The precision of the λ FLC assay was acceptable. CV's of <10% were observed for all serum pools and trueness of the control material assigned values verified.

3.1.5 Method comparison study

3.1.5.1 Correlation

Correlation describes the level of agreement between two methods and is a very important statistical tool that confers reliability of the regression statistics calculated.

For λ FLC, a correlation of 0.98 (r = 0.98) was observed indicating good agreement between the two methods as shown in figure 3.21.

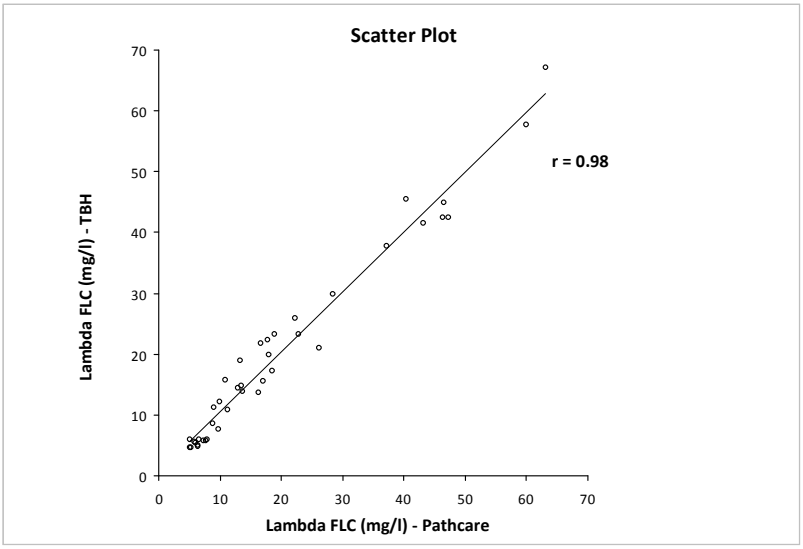

Figure 3.21 Method comparison correlation for λ FLC

For κ FLC, a correlation of 0.97 (r = 0.97) was observed indicating good agreement between the two methods as shown in figure 3.22.

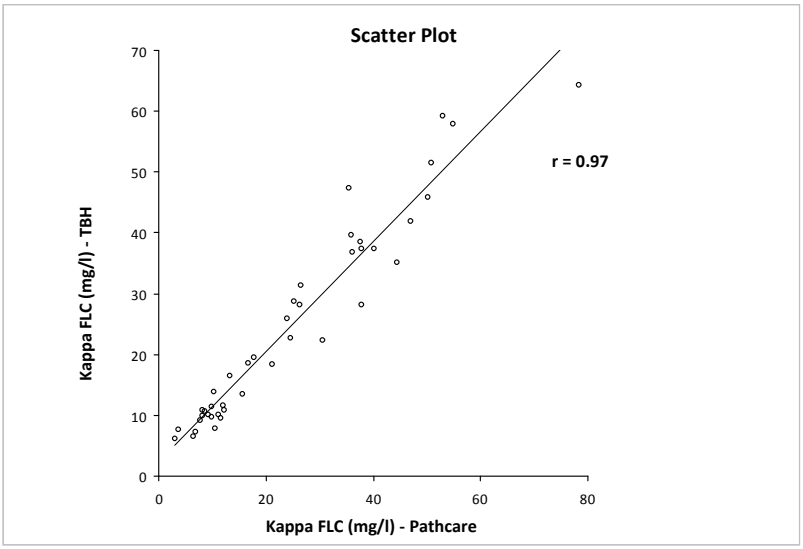

Figure 3.22 Method comparison correlation for κ FLC

3.1.5.2 Regression statistics

The λ regression equation calculated was y = 1.00x + 0.35; ($S_{y/x}$ =2.82 mg/l). The best-fit line demonstrated a constant error as shown in figures 3.23 and 3.24.

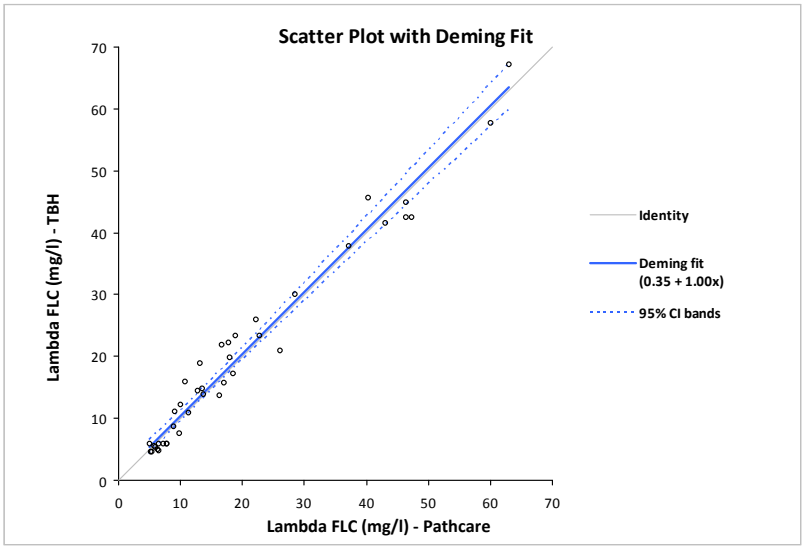

Figure 3.23 Deming regression statistics of λ FLC

The κ regression equation calculated was y = 0.93x + 1.71; ($S_{y/x}$ =4.32 mg/l). The best-fit line demonstrated both a constant and proportional error as shown in figure 3.24.

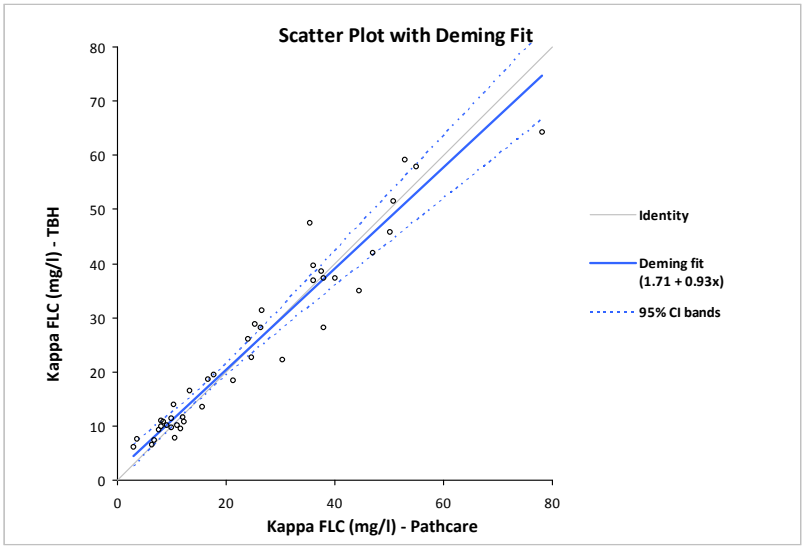

Figure 3.24 Deming regression statistics of κ FLC

3.1.5.3 Difference plots (Bland-Altman)

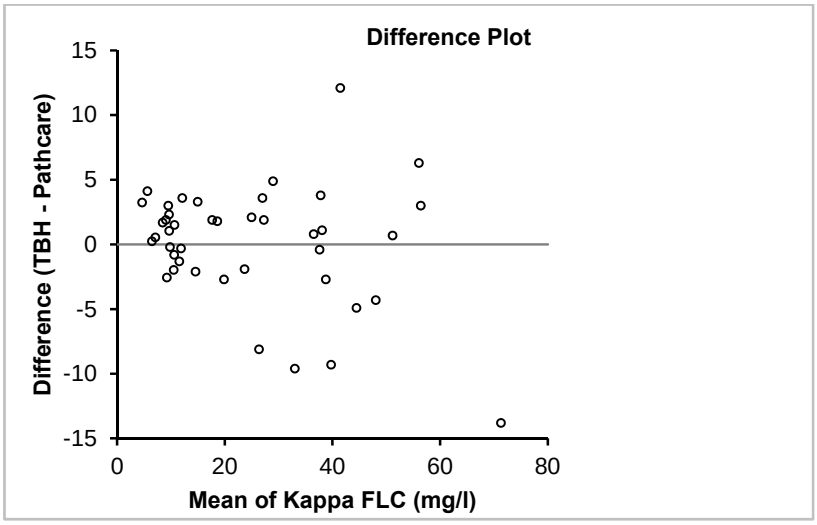

Figure 3.25 Difference plot for κ FLC

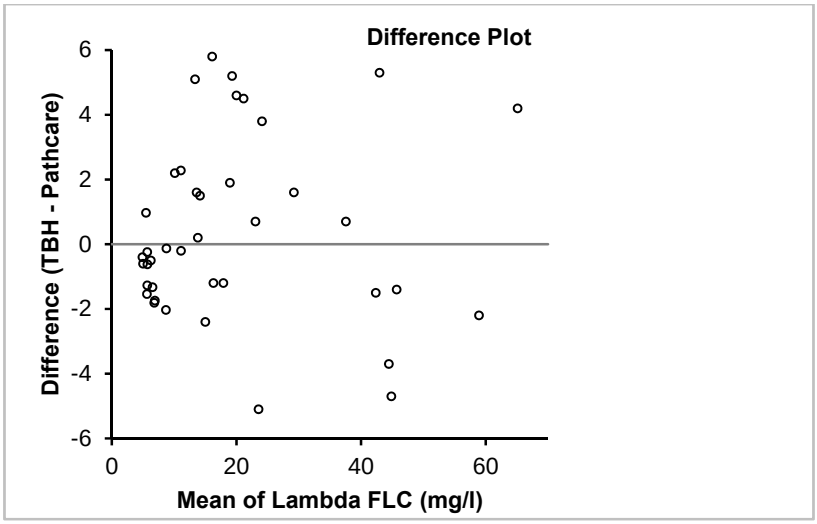

Figure 3.26 Difference plot for λ FLC

3.2 LOCAL REFERENCE INTERVALS

The population used for determining reference intervals consisted of 120 normal subjects, including 78 healthy HIV negative blood donors and 42 healthy black volunteers who were participating as controls in another study being performed by the Division of Haematology.

Their ages ranged from 19 – 60 years (median 36.5 years) with 83 (69%) females and 37 (31%) males (figure 3.25). The racial distribution was as follows: 12 (10%) whites, 48 (40%) mixed ancestry and 60 (50%) blacks (figure 3.26).

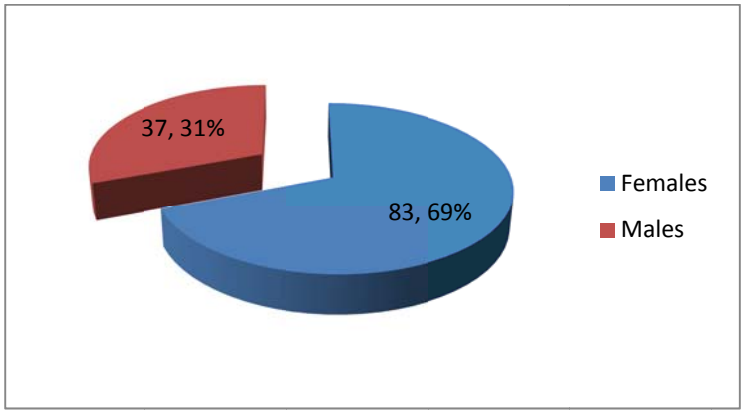

Figure 3.27 Gender distribution of 120 normal subjects

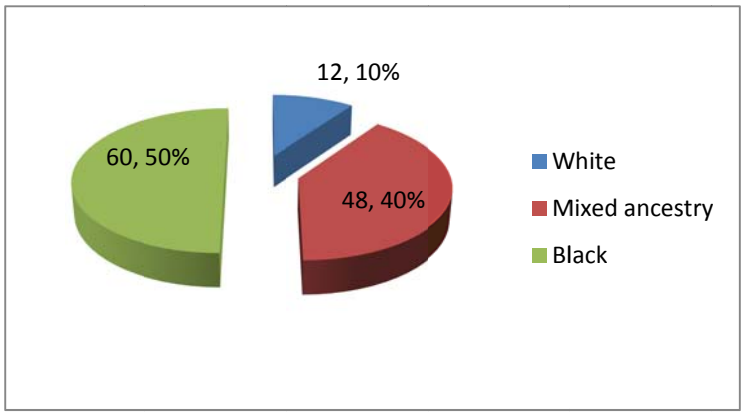

Fig 3.28 Racial distribution of 120 normal subjects

The serum creatinine was determined to see whether any of the subjects had renal impairment as this is known to affect the FLC values. All the creatinine values were in the normal range (44 – 116 µmol/l). Serum protein values were all within the normal range (61 – 92 g/L). The serum protein electrophoreses were independently reviewed by 3 pathologists. Most were normal, with a few polyclonal increases seen, but no definite monoclonal bands were detected. One patient had a hypogammaglobulinaemia on serum electrophoresis and a raised λ FLC and will be followed up. This sample was excluded from our analysis.

The 95% reference intervals obtained from our cohort using a non-parametric determination on Analyse-it® after elimination of outliers, as well as separate 95% reference intervals for Black and Mixed Ancestry subjects is shown in table 3.5.

Table 3.5 95% reference intervals for the various populations as compared to the manufacturer's recommendation

	MANUFACTURER'S RECOMMENDED 95% REFERENCE INTERVALS	LOCALLY DETERMINED 95% REFERENCE INTERVALS	LOCALLY DETERMINED 95% REFERENCE INTERVALS IN THE MIXED ANCESTRY POPULATION	LOCALLY DETERMINED 95% REFERENCE INTERVALS IN THE BLACK POPULATION
Kappa FLC (mg/l)	3.3 – 19.4	6.5 – 23.4	5.98 – 20.36	7.02 – 23.02
Lambda FLC (mg/l)	5.7 – 26.3	8.5 – 30.5	7.77 – 25.83	9.71 – 28.33
FLC ratio	0.26 – 1.2	0.5 – 1.2	0.5 – 1.2	0.5 – 1.3

Appendix 4 shows the graphs obtained for the 95% reference intervals on the Mixed Ancestry population.

Appendix 5 shows the graphs obtained for the 95% reference intervals on the Black population.

In conclusion, our reference values obtained were slightly higher than those recommended by the manufacturer. However, most values fell within the manufacturer's limits, and therefore we can accept the manufacturer's recommended cut-offs in our laboratory.

3.3 FREE LIGHT CHAINS IN HIV

A total of 369 HIV positive patients' serum from a previous study was analysed.

3.3.1 Characteristics of the study population

3.3.1.1 Demographics of the study population

Gender

Almost twice as many females as males were enrolled in this study, 127 males (34%) and 242 females (66%) as shown in figure 3.27.

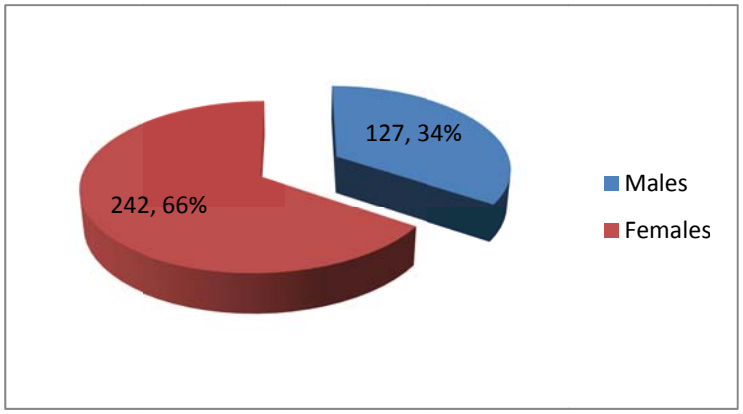

Figure 3.29 Gender distribution of 369 HIV positive patients

Age

The study population included patients from 17 to 70 years as shown in figure 3.28. The majority of patients were in the 25 – 54 age groups. The median age for the whole population was 37 years.

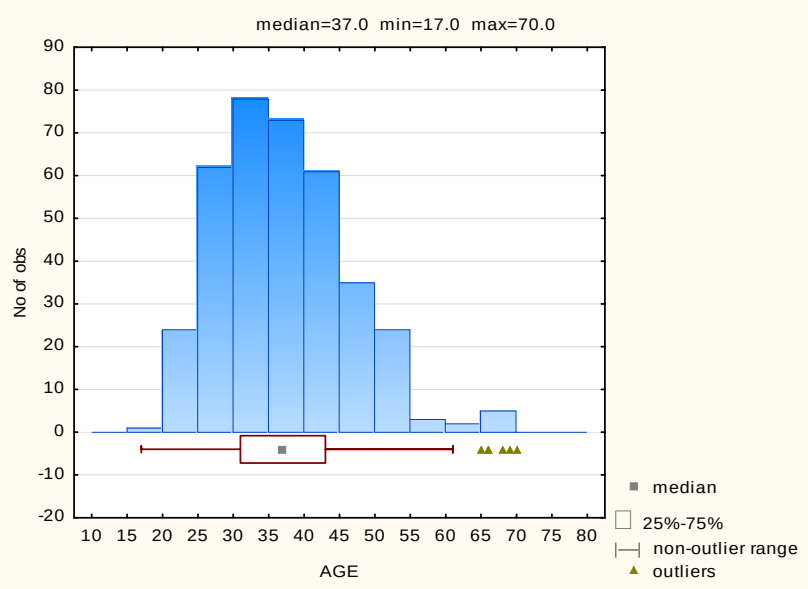

Figure 3.30 Age distribution of 369 HIV positive patients

Ethnicity

The population included patients from all the major ethnic groups resident in the Western Cape. The majority of patients were black (245, 66%), with 98 (26%) being of mixed ancestry. Only 6 (2%) of the patients were of Caucasian background, 6 (2%) were of unknown race and 14 (4%) were of other ethnic background as shown in figure 3.29.

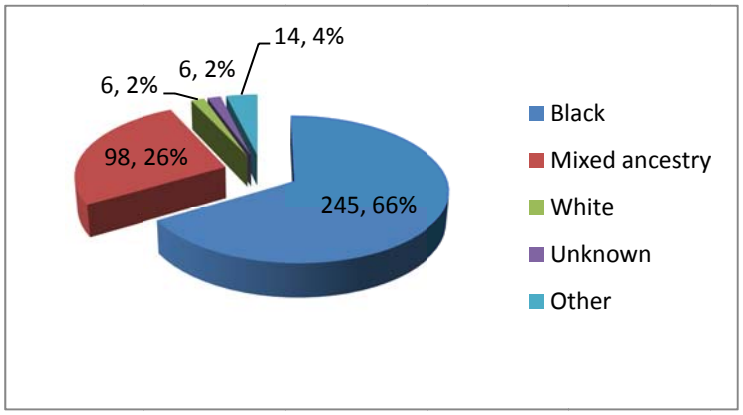

Figure 3.31 Ethnic composition of 369 HIV positive patients

Stage of Disease

The clinical stage of disease was documented for only 238 (64.5%) of patients. According to the WHO guidelines for HIV, 8 (4%) patients had stage I, 46 (19%) patients had stage II, 129 (54%) patients had stage III and 55 (23%) patients had stage IV HIV infection as shown in figure 3.30.

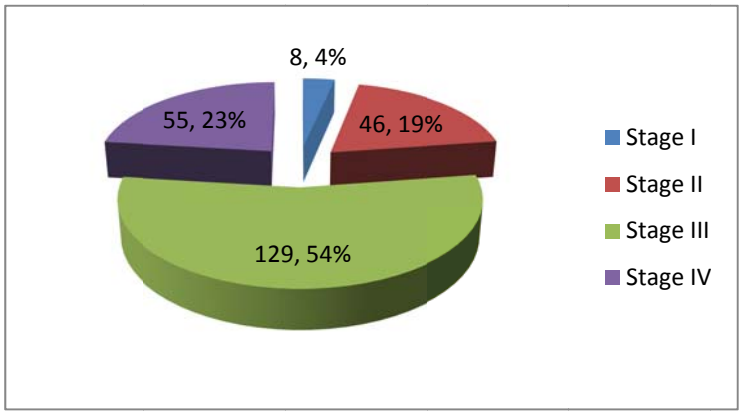

Figure 3.32 Stage of disease in 238 of the HIV positive patients

Use of ARV

A total of 327 (89%) patients included in the study were on ART as shown in figure 3.31. The duration of ART varied from 2 weeks to 13 years. The majority of patients however, had been on therapy for less than 60 months. The median duration of ART was 15.5 months.

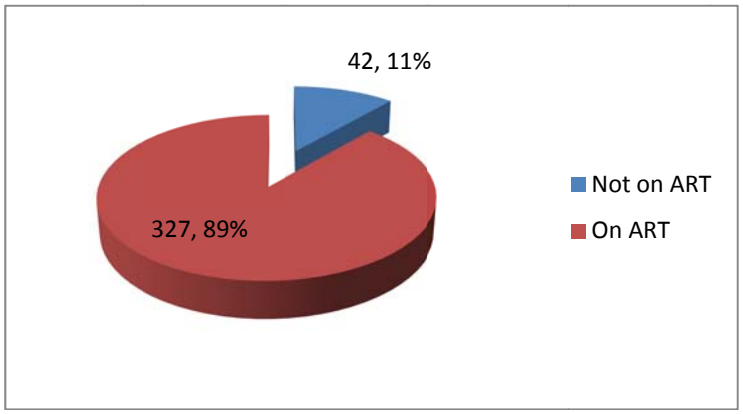

Figure 3.33 ART in 369 HIV positive patients

Co-existing medical conditions

Sixty-eight cases of co-existing medical conditions other than HIV infection were recorded. The majority of other medical conditions recorded were infection with *M. tuberculosis*. Forty-seven (69%) patients were on treatment for TB. Six (9%) had chronic pyogenic infections and 5 (7%) had a malignancy. Of these, 3 patients had Kaposi's sarcoma, 1 patient stage 4 cervical cancer and 1 patient had a recent mastectomy for breast carcinoma. No patients had severe liver disease documented. Two (3%) patients had a mild hepatitis, suspected to be alcohol or drug related. One patient had neurological conditions. Conditions rarely associated with HIV infection which was noted included 3 (4%) patients with diabetes mellitus. The 4 (6%) patients treated for arthritis were not diagnosed with any auto-immune arthropathy. One of them had gout. Figure 3.32 shows the co-existing medical conditions.

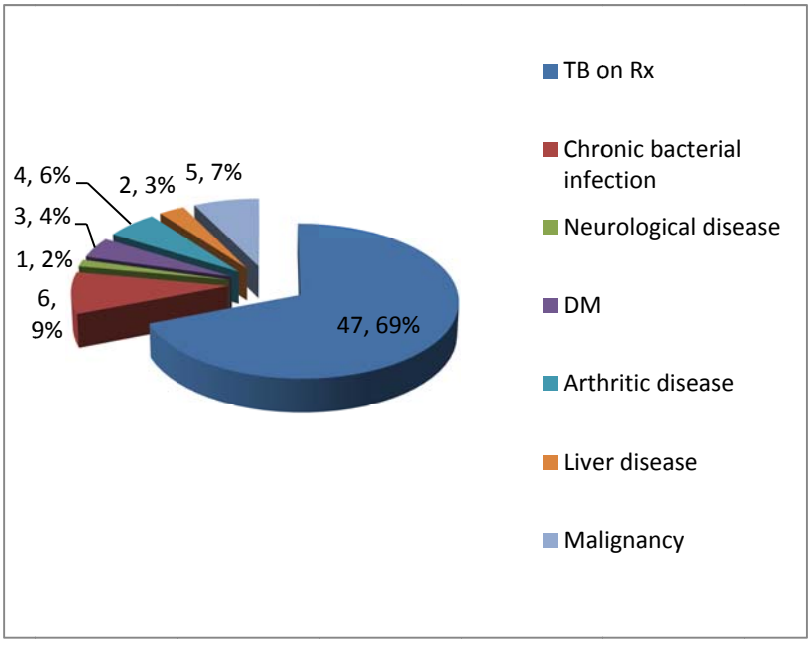

Figure 3.34 Co-existing medical conditions in 68 of the HIV positive patients

CD4 counts

CD4 counts were available for 353 (96%) of the 369 patients. The CD4 counts ranged from 1 to 1251 cells/mL. The median CD4 count was 287.5. The mean CD4 count increased with longer duration of therapy.

Viral load

Viral load results were available for 273 (84%) of the 327 patients on ART. ART suppressed viral replication successfully to undetectable levels in 228 (83.5%) patients, as measured by viral load detection. The lower limit of detection of the method used is 357 RNA copies/mL or 2.5 Log copies/mL. An additional 21 patients (7.6%) had a detectable viral load, but lower than the stated limit of detection. Only 24 (8.7%) patients had viral load results above the detection limit. No viral loads were available for patients not on ART.

Table 3.6 shows the median (and range) levels of the various tests performed on the study cohort.

Table 3.6 Results median (range) of tests performed on the HIV positive study cohort

VARIABLE	MEDIAN	RANGE
CD4 count (cells/ml)	287.5	1 – 1251
Total protein (g/l)	80.45	56.1 – 123.4
Albumin (g/l)	29.45	16.5 – 46.4
IgG (g/l)	22.12	9.57 – 112.9
IgA (g/l)	3.62	0.25 – 22.63
IgM (g/l)	1.4	0.4 – 89.0
Creatinine (µmol/l)	61.0	29.0 – 204.0

Gamma fraction

The gamma fraction as quantified using the Paragon™ Electrophoresis System and Appraise™ Densitometer System both from Beckman Coulter and varied in size from 8.2 to 80.6 g/L with a median value was 24.09 g/L. Although the range of values is wide, most patients had gamma fractions under 30 g/L. Only 82 (22%) of electrophoresis results had a gamma fraction of more than 30 g/L and 15 (4.9%) a gamma fraction of more than 40g/L. However, if the upper limits of the reference range (22 g/L) is considered, only 143 (39%) patients fall within the reference range.

Table 3.7 shows the median (range) values of κ and λ FLC's and the FLC ratio determined in the study cohort.

Table 3.7 FLC values in the HIV positive study cohort

VARIABLE	MEDIAN	RANGE
Kappa FLC (mg/L)	19.6	5.59 – 387.0
Lambda FLC (mg/L)	22.3	9.28 – 286.0
FLC ratio	0.85	0.13 – 3.28

Immunofixation

Electrophoresis was performed on 369 patients using agarose gel on the Paragon™ Electrophoresis System and Appraise™ Densitometer System both from Beckman Coulter. Immunofixation electrophoresis was performed on 100 (27%) of the 369 cases. The presence of a visible band on serum electrophoresis was the indication for immunofixation in only 27 (27%) of cases and no visible bands were found in 73 (73%) patients (figure 3.35). The majority of immunofixations were performed on the basis of a large gamma area where the presence of a visible band could not be excluded. Monoclonal bands were identified in 12 patients (3.2% of the total study population) after immunofixation and oligoclonal bands in a further 14 patients (3.8%).

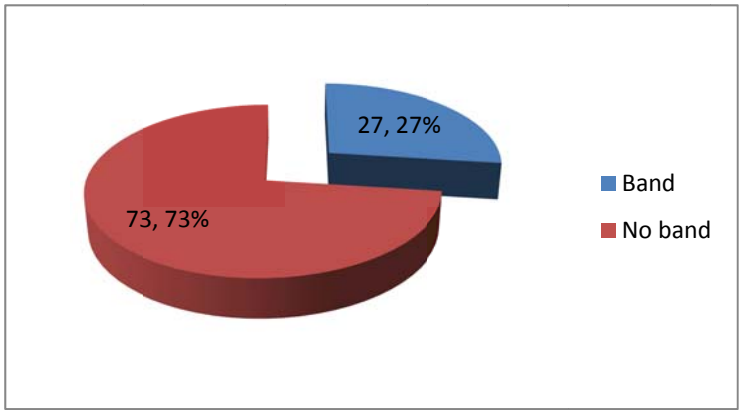

Figure 3.35 Results of Immunofixation (n = 100)

3.3.2 Correlations of FLC's with various variables

The Spearman rank correlation coefficient for non-parametric data was used to compare continuous variables to FLC's. Kappa and λ FLC's and FLC-ratio were compared to numerous variables. These included age, $CD4^+$ count, viral load, duration of disease, total protein values, albumin levels, gamma fraction levels, immunoglobulins levels and creatinine levels. Spearman correlations are not influenced by outliers and correlations are from -1 to +1, where +1 indicates a positive relationship and -1 a negative relationship. Values close to zero indicate no relation. The p-value tests the hypothesis that $r = 0$. A $p < 0.05$ rejects the correlation and is significant.

Age

Kappa and λ FLC and FLC-ratio were not significantly influenced by age (p= 0.8, p=0.6 and p=0.18 respectively).

$CD4^+$ count

Both κ and λ FLC showed significant inverse correlation with the $CD4^+$ count with the lowest $CD4^+$ counts showing high FLC levels ($p < 0.05$ for both). However FLC–ratio was not significantly influenced by $CD4^+$ count ($p = 0.18$). This is shown in Appendix 6

Viral load

We found a positive correlation between viral load and κ and λ FLC ($p < 0.05$ for both). However, the FLC-ratio was not influenced by viral load ($p = 0.65$).

Duration of disease

Kappa and λ FLC and FLC-ratio were all significantly negatively influenced by duration of disease. ($p < 0.05$ for κ and λ FLC and $p = 0.05$ for FLC-ratio). This is shown in Appendix 7.

Total protein

A significant positive correlation was found between κ, λ FLC and FLC-ratio and total protein values ($p < 0.05$ for all).

Albumin

A significant negative correlation was found between albumin levels and κ and λ FLC. ($p < 0.05$). However there was no significant correlation between albumin levels and FLC-ratio ($p = 0.6$).

Gamma fraction

A significant positive correlation was found between the gamma fraction and κ and λ FLC and FLC-ratio. ($p < 0.05$ for all). This is shown in Appendix 8.

IgG

A significant positive correlation was found between the IgG level and κ and λ FLC and FLC-ratio. ($p < 0.05$ for all). This is shown in Appendix 9.

IgA and IgM

A significant positive correlation was found between the IgA level and κ and λ FLC. ($p < 0.05$ for both). However the correlation between IgA and FLC-ratio was not significant ($p = 0.1$). A similar picture was seen for IgM.

Creatinine

A slight positive correlation was found between the creatinine levels and κ and λ FLC. ($p = 0.01$ and $p = 0.28$ respectively). However, significantly positive correlation was found between creatinine levels and the FLC-ratio ($p < 0.05$).

Table 3.8 shows the correlation of κ and λ FLC's and FLC ratio to the various variables.

Table 3.8 Correlation of κ and λ FLC's and FLC ratio to various variables ($p<0.05$ significant)

VARIABLES	KAPPA FLC		LAMBDA FLC		FLC RATIO	
	Spearman correlation (r-value)	p-value	Spearman correlation (r-value)	p-value	Spearman correlation (r-value)	p-value
Age	-0.01	0.80	0.03	0.6	-0.07	0.18
CD4 count	-0.34	<0.05	-0.34	<0.05	-0.07	0.18
Viral load	0.21	<0.05	0.22	<0.05	0.03	0.65
Duration of disease	-0.43	<0.05	-0.35	<0.05	-0.10	0.05
Total protein	0.43	<0.05	0.37	<0.05	0.17	<0.05
Albumin	-0.17	<0.05	-0.21	<0.05	0.03	0.60
Gamma	0.58	<0.05	0.51	<0.05	0.24	<0.05
IgG	0.66	<0.05	0.61	<0.05	0.23	<0.05
IgA	0.19	<0.05	0.29	<0.05	-0.09	0.10
IgM	0.37	<0.05	0.41	<0.05	0.00	0.96
Creatinine	0.13	0.01	0.06	0.28	0.15	<0.05

3.3.3 Relationships of FLC's with non-continuous variables

ANOVA analysis was used to correlate κ and λ FLC and FLC-ratio to non-continuous variables, such as gender, ethnicity, stage of disease, use of ART's and the presence of an abnormal serum protein electrophoresis. The data was first log transformed to minimize the effect of outliers on the results. The F-test and the Mann-Whitney U test were used. They test the hypothesis that the means are equal. A p-value of < 0.05 rejects the hypothesis and is significant.

Gender

Kappa and λ FLC and FLC-ratio were slightly higher in males than in females. This was only significant for κ FLC ($p = 0.02$), but not significant for λ FLC ($p = 0.13$) and FLC ratio ($p = 0.45$).

Ethnicity

No significant difference was found in κ and λ FLC and FLC ratio between those of mixed ancestry and black ($p = 0.25$, $p = 0.16$ and $p = 0.26$ respectively).

Stage of disease

There seemed to be an upward trend between stage of disease and κ and λ FLC ($p = 0.06$) and ($p < 0.01$) respectively. However, there was no significant trend between FLC ratio and stage of disease ($p = 0.37$). This is shown in Appendix 10.

Use of ART's

A strongly significant difference was seen between κ FLC values in patients taking ART'S compare to those not ($p < 0.01$). Those taking ART'S had lower levels of κ FLC. The same was found when comparing these two groups with λ FLC values ($p < 0.01$).

However, FLC ratio was not significantly affected by ART use ($p = 0.98$). This is shown in Appendix 11.

Immunofixations for abnormal serum protein electrophoresis

Kappa and λFLC and FLC ratio values differed significantly when an abnormal band was found on serum protein electrophoresis ($p < 0.01$for all). This is shown in Appendix 12.

SECTION IV: DISCUSSION

4.1 METHOD VALIDATION

It is a standard process to validate a new method before its introduction as part of good laboratory practice. Method validation focuses on analytical errors and the impact of these errors on the interpretation of the test.

Dr Westgard refers to method validation simply as "*error assessment*". The focus is on analytical errors and how these errors impact on the interpretation of a test. While a comparison of method experiment can reveal the different type of errors, there are specifically designed experiments for each one of them. (Westgard. 2008) Random errors can be detected using replication studies, constant errors using interference studies and method comparison studies and proportional errors using recovery, linearity and method comparison studies.

Serum FLC assays are not without problems (Tate et al. 2009). Experienced staff, proper laboratory techniques, and well maintained equipment is important for serum FLC assays (Davids et al. 2010). Batch to batch inconsistency (Davids et al. 2010), antigen excess causing underestimation of high results (Davids et al. 2010; Bosmann et al. 2010; Daval et al; 2007), risk of overestimation and overestimation with dilutions (Tate et al. 2007) and polymerization of FLC can lead to false overestimation (Tate et al. 2003; Bradwell et al. 2001; Abrahams et al. 2002). Measurements of κ and λ FLC should be performed in the same laboratory with the same analyzer (Tate et al. 2003), because different results may be obtained with different analyzers, thus each lab should estimate its own local reference ranges. (Briand et al. 2010; Beetham et al. 2007).

We found κ and λ FLC's to be linear within the manufacturer's recommended range using both linearity fluid and pooled serum obtained from routine samples received in the chemical pathology laboratory. Recovery studies were performed to estimate the effects of specific materials on the accuracy of the method or systematic error. The average λ recovery was excellent, but we found the κ recovery to be only 40.36%, showing a large proportional error, probably due to matrix effect. We consulted the Binding Site about

this result, but were told that as the rest of the method validation study was acceptable, we could accept this as matrix effect and introduce the test.

Interferences studies were performed to estimate constant error caused by Hb due to haemolysis, bilirubin, rheumatoid factor and triglycerides interfering with the assay. When studying the effect of haemolysis on λ FLC results using both low and high pools, we found that results were within the 10% allowable error, but the κ FLC high pool was out of the 10% allowable laboratory error, most likely due to a random error. Again we contacted the Binding Site and were told we could introduce the test, as the results were within a 20% allowable error. Bilirubin, rheumatoid factor and triglyceride interference studies did not exceed the 10% or 20% allowable error for κ and λ FLC. In fact, all the results were within the 10% allowable error, showing minimal interference for both κ and λ FLC.

Imprecision studies were performed using the CLSI EP-15 protocol to establish the amount of random error (Chesher. 2008). The precision of κ FLC was acceptable. Trueness of the assigned control material was verified and acceptable CV's of <10% were observed for all κ serum pools. The precision of λ FLC was acceptable. When trueness of the assigned control material was verified, CV's of <10% were observed for all serum pools.

Method comparison studies estimates bias, for example the inaccuracy or systematic error of a method. We compared the results obtained on our Beckman IMMAGE^{TM} analyzer to those obtained on the same instrument at a local private laboratory. Correlation studies between the two laboratories for κ and λ FLC showed a correlation of 0.97 (r=0.97) and 0.98 (r = 0.98) respectively, indicating an excellent agreement between the two methods. The λ regression equation calculated was y = 1.00 x+ 0.35 with the best-fit line demonstrating a constant error. The κ regression equation was y = 0.93 x+ 1.71 with the best-fit line demonstrating both a constant and proportional error.

4.2 LOCAL FLC REFERENCE RANGES

Serum FLC assays are an important diagnostic, prognostic, therapeutic marker and management tool in a variety of diseases such as MM with its phenotypes(Davids et al. 2010; Kyrtsonis et al. 2007; van Rhee et al. 2007), NSMM (Drayson et al. 2001), light chain disease (Bradwell et al. 2003; Katzmann et al. 2006) , MGUS (Rajkumar et al. 2005), solitary bone plasmacytoma (Dingli et al. 2006), smouldering myeloma (Dispenzieri et al. 2008), Al amyloidosis (Katzman et al. 2005), Waldenström's macroglobulinaemia (Izykson et al. 2008; Leleu et al. 2008)] and chronic lymphocytic leukemia (Martin et al. 2007).

Although manufacturers 95% reference intervals have been established (Katzmann et al. 2002), we decided to establish our own reference intervals, as part of good laboratory practice. Besides this being part of good laboratory practice, we also felt it necessary to establish local reference intervals, as the manufacturers' reference intervals were established mainly on an older Caucasian population. Our study population age and race distribution is not the same as we studied mainly younger black and mixed ancestry subjects.

Tate and Pattenden described in their respective studies that different FLC values were obtained on different analyzers when analyzing the same sample (Tate et al. 2007; Pattenden et al. 2007). Therefore, laboratories should validate FLC reference intervals on their assay using at least 20 normal samples to confirm that at least 90% of the sample values fall within the recommended reference interval for FLC concentration and ratio (Tate et al. 2009).

Several studies have shown differences in the prevalence of MGUS and MM in various ethnic groups. In a study by Weiss et al was it reported that FLC values are higher in blacks with MGUS than in Caucasians (Weiss et al. 2011). The prevalence of MGUS and MM are higher in African Americans compared to Caucasians with Asians having the lowest prevalence, which points to a definite difference in ethnic and genetic factors

contributing to the development of MGUS and MM (Landgren and Weiss. 2009). Katzmann et al described an increase in FLC concentration with increasing age, with a dramatic increase in those older than 80 years. This has been postulated to be due to renal impairment associated with age. This is supported by the fact that the κ/λ FLC ratio does not increase with age-dependant increases in FLC (Katzmann et al. 2002). Therefore we postulated that FLC levels may be higher in our population due to their ethnicity, but as their age was younger, the levels would be less influenced by age-related decline in renal function.

Our study population was younger with a median age of 37 years and 66% were of black ethnicity. We used 120 healthy HIV negative subjects consisting of healthy blood donors and healthy control subjects partaking in another study. The demographics were similar to our study population with an age range of 19 – 60 years (median 36.5 years). The racial distribution was as follows: 12 (10%) Caucasians, 48 (40%) mixed ancestry and 60 (50%) blacks. Serum creatinine was determined to exclude renal impairment as this affects the FLC values (Katzmann et al. 2002). All the creatinine values fell within the normal range of 44 – 116 µmol/l. We also performed serum protein electrophoresis on all subjects to exclude abnormal FLC values due to undiagnosed plasma cell dyscrasias.

When mixed ancestry and black subjects were analyzed separately, data was first log-transformed and then analyzed. The local reference interval of the black subjects in our cohort is in agreement with results from Weiss *et al* that showed that black MGUS patients had higher FLC levels (Weiss et al. 2011). Our reference intervals were slightly higher than those recommended, however as most of our values fell within the manufacturers limits, it was decided to accept these cut-offs in our study after consultation with the Binding Site, as most results still fell within the recommended cut-offs.

4.3 FREE LIGHT CHAINS IN HIV POSITIVE SUBJECTS

The main purpose of this study was to investigate whether FLC's as a marker of B-cell dysfunction are increased in subjects with HIV. FLC are known to be markers of B-cell dysfunction and as subjects with HIV are described to have B-cell abnormalities, we hypothesized that FLC levels and FLC ratio will be abnormal in HIV-infected individuals. Despite B-cell dysfunction being described in HIV (De Milito. 2004; Sodora and Silvestri. 2008; Moir and Fauci. 2009; Moir and Fauci. 2008; Caggi et al. 2008; Virgin and Walker. 2010; Bussmann et al. 2009; Appay and Sauce. 2008), there is a paucity of literature available on FLC levels in HIV (Landgren et al. 2010).

Our cohort consisted of 369 HIV positive subjects, many of whom were on HAART. The cohort was judged to be representative of the patients attending clinics for ART and disease management in the Western Cape. The study population included 127 males (34%) and 242 females (66%). There were twice as many females than males that were enrolled in the study, but is in agreement with what is expected, based on recently available HIV prevalence rate estimated for the Western Cape (Department of Health. 2007). The Human Science Research Council (HSRC) report also cited a higher prevalence in females in South Africa (13.3% in females vs 8.2% in males) (www.hsrcpress.ac.za). The study population age ranged from 17 to 70 years, with most between 25 to 54 years. The median age for the whole study population was 37 years, with males being significantly older than females. The peak age prevalence of HIV infection in South Africa is between the ages of 25 and 34 years for females and 30 to 39 years for males. The age difference is therefore expected. The mean age of patients attending the clinic would thus be expected to be slightly older than the peak prevalence age, due to the time it takes for diseases progression before treatment becomes necessary (www.hsrcpress.ac.za).

The population included patients from all the major ethnic groups resident in the Western Cape. The majority of patients were black (245, 66%), with 27% (98) being of mixed ancestry. Only 6 (2%) of patients in the study population was of Caucasian background,

6 (2%) of the patients in the study were of unknown race and 14 (4%) were of other ethnic background. The largest population group in the Western Cape is of mixed ancestry (54%), followed by Blacks (27%), Caucasians (18%) and Indian/Asians (1%) (www. Statssa.gov.za). The prevalence rates of HIV differs between population groups in South Africa (Blacks 13,3%, Mixed Ancestry 1,9% Indian/Asian 1,6% and Whites 0,6%) (www.hsrcpress.ac.za). The ethnic composition of patients attending HIV clinics in the Western Cape would therefore be expected to approximately be 76% Blacks, 22% Mixed Ancestry, 2% Caucasian and less than 1% Asian/Indian. Our study population is therefore representative of the population groups expected to be seen at the clinics.

The clinical stage of disease was documented for only 238 (64.5%) of patients. According to the WHO guidelines for HIV, 8 (3%) patients had stage I, 46 (19%) patients had stage II, 129 (54%) patients had stage III and 55 (23%) patients had stage IV HIV infection. Tuberculosis (TB) is endemic in the Western Cape with an incidence rate of 1 041/100 000 of the population reported in 2005 (Bradshaw et al. 2005). Of the 68 patients in our cohort having co-existing medical conditions, 47 (69%) were being treated for TB.

According to Moir and Fauci, decreased $CD4^+$ count and not viral load, leads to increased immature B-cells in HIV (Moir and Fauci. 2008). CD4 counts were available for 353 (96%) of the 369 patients and ranged from 1 to 1251 cells/mL with a median value of 287.5 cells/mL. The mean CD4 count increased significantly with increased duration of ART. Both κ and λ FLC showed a significant inverse correlation with $CD4^+$ counts with the lowest $CD4^+$ counts having higher FLC levels. This correlation supports the theory of B-cell dysfunction as a feature of HIV pathogenesis and may be a valuable marker of potential disease progression.

Viral load results were available for 273 (84%) of the 327 patients on ART. Ideally, viral loads should be decreased to < 400 RNA copies/ml with effective ART. ART suppressed viral replication successfully to undetectable levels in 228 (83.5%) patients, as measured by viral load detection. The lower limit of detection of the method used is 357 RNA

copies/mL or 2.5 Log copies/mL. An additional 21 patients (7.6%) had a detectable viral load, but lower than the stated limit of detection. Only 24 (8.7%) patients had viral load results above the detection limit. We found a positive correlation between viral load and κ and λ FLC. However FLC-ratio was not influenced by viral load which supports the well described finding of polyclonal B-cell activation in HIV infection.

The serum total protein values ranged from 56.1 to 123.4 g/L with a median value of 80.45 g/L, which is indicative of some of these subjects having increased protein levels, most likely due to increased gamma fractions. Albumin levels were quantified using the Paragon™ Electrophoresis System and Appraise™ Densitometer System both from Beckman Coulter and ranged from 16.5 to 46.4 g/L, with a median of 29.45 g/L. Albumin is a negative acute phase reactant and decreases in the acute phase of infection. We found a significantly negative correlation between albumin, and κ and λ FLC levels, which indicates that B-cell activation and decreased albumin occurs in the acute phase of HIV infection. Again there was no significant correlation between albumin and FLC ratio, pointing to polyclonality.

The gamma fraction was also quantified using the Paragon™ Electrophoresis System and Appraise™ Densitometer System and varied in size from 8.2 to 80.6 g/L with a median value was 24.09 g/L. Although the range of values is wide, most patients had gamma fractions under 30 g/L. If the upper limit of the reference interval (22 g/L) is considered, 143 (39%) patients fell within the reference range. A strong positive correlation was found between the gamma fractions and κ and λ FLC values and κ /λ FLC ratio. As the gamma fraction is most likely indicative of increased immunoglobulins, this correlation again points to B-cell hyperactivity.

As the gamma fraction was determined densitometrically and is therefore more of a subjective measurement, the IgG value which is determined nephelometrically, is more reliable. IgG levels varied from 9.57 – 112.9 g/L with a median value of 22.12 g/L. A strong positive correlation was found between IgG levels and κ and λ FLC values and κ /λ FLC ratio. This is self-explanatory, as FLC levels will be increased with increased

immunoglobulin synthesis and these were thus expected. A similar positive correlation was found between IgA and IgM levels and κ and λ FLC values, but not for κ /λ FLC ratio, pointing to polyclonal IgA and IgM synthesis.

As FLC are known to be influenced by renal function, we determined creatinine levels and these varied from 29.0 – 204.0 µmol/L with a median value of 61.0 µmol/L. A slightly positive, but still statistical significant correlation was found between creatinine and κ and λ FLC values. This is to be expected as FLC are filtered and excreted by the kidney, so higher creatinine levels indicating decreased renal function would result in higher FLC values. Though the literature describes these subjects as having FLC ratio values within the reference intervals (Katzmann et al. 2002), we found a significant positive correlation between FLC ratio and creatinine levels in our study.

FLC values and FLC ratio were also correlated to non-continuous variables after log transformation. Kappa and λ FLC values were found to be slightly higher in males than in females in our study cohort; however 34% of the cohort was males who were older, which perhaps attributed to this. As expected in HIV, some of our subjects with HIV had high κ and λ FLC values with κ FLC ranging from 5,59 – 387,0 mg/L with a median of 19,6 mg/L and λ FLC ranging from 9,28 – 286,0 mg/L with median of 22,3 mg/L.

When correlating κ and λ FLC and κ/ λ FLC ratio to age, no significant correlations were found. This is contradictory to what is found in the literature which shows increased values with age most likely due to decreasing renal function (Katzmann et al. 2002). No significant difference was found in κ and λ FLC and FLC ratio between those of mixed ancestry and Black origin.

Of great significance was the statistically significant inverse correlation that was found between $CD4^+$ counts and κ and λ FLC levels. Traditionally, HIV has been associated with $CD4^+$ count abnormalities and cellular immunity dysfunction (Schnittman et al. 1986; Lane et al. 1983). Recent publications have shown marked B-cell dysfunction in HIV and this may be due to the T-cell effect on B-cell function (Caggi et al. 2008; de

Milito. 2004; Moir and Fauci. 2008; Sodora and Sivestri. 2008; Moir and Fauci. 2009; Virgin and Walker. 2010; Bussmann et al. 2009; Appay and Sauce. 2008). However the FLC ratio was not influenced by $CD4^+$ count which points to polyclonal B-cell activation. These results support the finding that T-cells have an important influence on B-cell function.

Another exciting finding was the significant positive correlation between viral load and κ and λ FLC levels, but not the ratio. This again points to increasing B-cell dysfunction with disease severity. The fact that FLC ratio is not significantly influenced by $CD4^+$ and viral load, points to polyclonal B-cell dysfunction.

When examining the stage of disease and its correlation with κ and λ FLC values and FLC ratio, a definitive upward trend was seen between these two variables. This was only evident from stage 2 onward, but it must be noted that only 8 subjects had stage 1 disease. There was no correlation between FLC ratio and stage of disease, again pointing to polyclonality.

Kappa and λ FLC as well as FLC ratio negatively and significantly influenced by duration of disease, most likely as a result of the commencement of treatment in these subjects with recovery of B-cell dysfunction.

As mentioned previously, this cohort was used in a previous study to determine the prevalence of monoclonal band in HIV positive subjects in our local population, so serum protein electrophoresis was performed on the subjects (Jansen van Vuuren et al. 2010). Immunofixation electrophoresis was performed on 100 (27%) of the 369 cases for an abnormal pattern on serum protein electrophoresis. A visible band was seen in only 27 (27%) of these cases and no visible bands were seen in 73 (73%). The majority of immunofixations were performed due to a large gamma area where the presence of a visible band could not be excluded. Monoclonal bands were identified in 12 patients (3.2% of the total study population) after immunofixation and oligoclonal bands in a further 14 patients (3.8%). When correlating κ and λ FLC values and FLC ratio to the

presence of an abnormal band in serum protein electrophoresis, the presence of an abnormal band was associated with significantly higher κ and λ FLC values and FLC ratio. This supports the fact that FLC values are increased in conditions associated with an abnormal serum protein electrophoresis, such as plasma cell dyscrasias.

When correlating the use of ARV'S with κ and λ FLC values, a significant difference was found between those taking ARV'S and those not. Kappa and λ FLC values were significantly higher in the group not on treatment. This is to be expected and supports the theory that ARV'S improve B – cell dysfunction in HIV positive subjects. No correlation was found between the use of ARV'S and FLC ratio, again supporting polyclonality.
There are several limitations to the study:

Firstly, albumin and gamma fractions were determined densitometrically. This technique is subjective and dependant on technologist expertise, however most of the serum protein electrophoreses were performed by the same technologist, so there should have been continuity. However, IgG values were determined and they correlated well with the gamma fractions.

Secondly, creatinine was used as a marker of renal function. However, creatinine is not a reliable marker of renal function and only increases late when up to 50% of renal function been lost. A better test of renal function would have been a creatinine clearance, but this is a cumbersome test, requiring a 24-hour urine sample. Cystatin C has also been described as a good marker of renal dysfunction (Katzmann et al. 2002), but unfortunately the test is not available in our laboratory, so we had to rely on creatinine.

Thirdly, viral loads, CD4+ counts and immunofixation results were not available for all 389 subjects.

Fourthly, only 8 subjects had stage I disease and therefore the results correlating FLC values and FLC ratio to stage of disease are unreliable for stage I.

Finally, three years after the initial study (Jansen van Vuuren et al. 2010) was performed on this cohort, we have not yet received one follow-up sample on abnormal results, despite numerous communications to the clinicians. This signifies a total loss of follow-up in this cohort. Landgren et al. described FLC as a prognostic marker in HIV for future malignancies (Landgren et al. 2010). However, we will not be able to verify this in our cohort due to the loss of follow-up.

SECTION V: CONCLUSION

CONCLUSION

Method validation of the FLC assay on the Beckman IMMAGE® was found to be acceptable for our study and introduction for routine use in our laboratory is advocated.

Although we postulated that the reference intervals in our population would differ from the manufacturers' recommended reference intervals due to the difference in study population, we found that we could accept the recommended reference intervals in our population.

As expected, FLC which is a marker of B-cell dysfunction was increased in subjects with HIV. Traditional markers of HIV severity, namely CD4+ T-cell count and viral load correlated with these levels. The use of ARV, which is known to improve B-cell dysfunction in HIV, led to decreased FLC levels indicating that the B-cell function is indeed improved in these subjects.

We would like to have studied the future effects of these raised and abnormal FLC values in these subjects to see if they had a worse prognosis and maybe higher incidence of B-cell malignancies in the future, but as mentioned in the limitations of this study, most of this cohort seems to be unfortunately lost to follow-up.

A future study is already being planned following on from this, where we will examine at FLC values in a cohort of newly diagnosed treatment-naïve HIV positive subjects. We will then follow these subjects up to see if they indeed have a worse prognosis and further study the effect of ART on the FLC values.

SECTION VI: BIBLIOGRAPHY

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APPENDIX 1

ADDENDUM A:

CLINICAL DATA: Study on monoclonal gammopathies in patients with HIV

Study patient identification number_____

Date:_____

Demographics:

Age: _____

Gender: M / F

Disease stage:

WHO clinical stage:_____

Latest

- CD4_____ Date_____

- Viral load_____ Date_____

Other disease diagnosis: (pls indicate diagnosis and treatment where appropriate)

- TB: Current/ Previous_____

  Treatment:_____

- Auto-immune disease: Diagnosis _____

  Treatment: _____

- Malignancy: Diagnosis_____

  Treatment: _____

- Viral hepatitis : Diagnosis _____

  Treatment: _____

- Other: Diagnosis _____

  Treatment: _____

Treatment:

HAART: Y / N

Date started: _____

1st /2nd regimen:

Previous regimen if applicable: _____

Current regimen: Drugs: (Circle pls)

d4T	3TC	EFV	Other _____
AZT	ddi	NVP	
ABC		KLT	
TDF		SQV	
		RTV	

Person filling in this form:

Name _____ Signature_____

ADDENDUM B:

DEELNEMERS INLIGTINGSBLAD EN TOESTEMMINGSVORM

Titel van navorsingsprojek:
Monoklonale pieke in pasiënte met HIV: Die prevalensie en assosiasie met stadium van siekte, chroniese antigeen stimulasie en die effek van antiretrovirale terapie.

En

Vergelyking van die sensitiwiteit en spesifisiteit van agarose gel elektroforese, kapillêre sone elektroforese en serum vry ligte ketting analise in die diagnose van monoklonale gammopatie in pasiënt met HIV.

Verwysingsnr:
Hoofnavorser: Dr A Zemlin
Adress: Afdeling Chemiesie Patologie, NHLS, Tygerberg Hospitaal.

- U word genooi om deel te neem aan 'n navorsingsprojek. Lees asseblief hierdie inligtingsblad op u tyd aangesien die besonderhede van die navorsingsprojek daarin verduidelik word.

- Hierdie navorsingsprojek is deur die Komitee vir Mensenavorsing van die Universiteit Stellenbosch goedgekeur en sal uitgevoer word volgens die etiese riglyne en beginsels van die Internasionale Verklaring van Helsinki en die Etiese Riglyne vir Navorsing van die Mediese Navorsingsraad (MNR).

- Die navorsingsprojek behels die volgende:
  Die doel van die projek is om te kyk na die antiliggaamprofiel van pasiënte met HIV infeksie op serum elektroforese. Ons is veral geinteresseerd in die teenwoordigheid van monoklonale pieke. Dit is die teenwoordigheid van 'n band op die serum elektroforese wat aanduidend is van 'n enkele tipe antiliggaam wat deur 'n kloon van antiliggaamproduserende selle gemaak word. Elektroforese is 'n metode wat gebruik word om proteine in die bloed van mekaar te skei. Die monoklonale pieke kom ook voor in pasiënte wat nie HIV infeksie het nie waar dit dikwels dui op die teenwoordigheid van 'n limfoom of veelvuldige miëloom ('n kanker van antiliggaamproduserende selle) of die ontwikkeling daarvan voorgaan. Hierdie pasiënte word noukeurig opgevolg vir die ontstaan van die toestande. Monoklonale pieke kom meer algemeen voor wanneer 'n persoon HIV infeksie het, maar die betekenis daarvan is nie duidelik nie. Dit mag moontlik net die gevolg wees van die immuunrespons teen die virus of mag moontlik 'n risiko wees om limfoom of veelvuldig miëloom te ontwikkel. Ons wil die betekenis van die monoklonale pieke verder ondersoek om riglyne daar te stel vir die hantering en opvolg daarvan.

- **Dieselfde** monsters sal ook met verskillende metodes (agarose gel elektroforese, kapillêre sone elektroforese en serum vry ligte kettings) geanaliseer word om vas te stel watter metode die mees sensitief en spesifiek is sodat pieke nie gemis word nie,
  maar onnodige verdere ondersoeke nie gedoen word nie. Hierdie data sal ook gepubliseer word as 'n aparte studie.

- Die studie behels die neem van bloed (10ml/ 2 teelepels) en 'n uriene monster(100ml / halwe koppie).

- Indien daar wel ‘n monoklonale piek teenwoordig is sal u die nodige verdere ondersoeke aangebied word om die teenwoordigheid van limfoom of plasma sel tumore uit te skakel. Dit mag moontlike X-strale, CT skandering of beenmurgbiopsie behels. Dit sal met u bespreek word en toestemming verkry word vir die ondersoeke indien nodig. Die piek sal ook 6 maandeliks of jaarliks opgevolg word met ‘n bloed of uriene monster.

- Alle pasiente met HIV infeksie wat opvolg word by die Tygerberg Infeksie Siekte Kliniek word genooi om aan die studie deel te neem.

- Deelname is vrywillig en dit staan u vry om deelname te weier. U mag ook enige tyd aan die projek onttrek, selfs al het u ingestem om deel te neem.
  Deelname sal nie die behandeling wat u ontvang beïnvloed nie.
- Die voordeel vir u om aan die studie deel te neem is dat die teenwoordigheid van ‘n monoklonale piek geïdentifiseer word en dat u opgevolg sal word.
  Die risiko’s verbonde aan deelname behels die normale risiko’s van bloedtrek, nl
  - pyn by die area waar bloed getrek word
  - bloeding in en rondom die area waar bloed getrek is of hematoom vorming.

  Indien verdere opvolg ondersoeke nodig is sal die prosedure en risiko’s aan u verduidelik en u toestemming verkry word.

- Al die inligting wat versamel word sal vertroulik hanteer word. Dit sal gebruik word in ‘n publikasie en tesis, maar die identiteit van deelnemers sal anoniem bly. Die mediese personeel wat u inligting versamel en die navorsers wat dit prosesseer sal toegang hê tot die inligting.

- U sal nie betaal word om deel te neem aan die projek nie, maar dit sal u ook niks kos nie.

- Enige vrae kan aan die dokter wat u behandel gerig word of die navorsers kan gekontak word by die bogenoemde nommers.

Verklaring deur deelnemer

Ek_____ stem in om deel te neem aan die navorsingsprojek getiteld:
Monoklonale pieke in pasiënte met HIV: Die prevalensie en assosiasie met stadium van siekte, chroniese antigen stimulasie en die effek van antiretrovirale terapie.
En
Vergelyking van die sensitiwiteit en spesifisiteit van agarose gel elektroforese, kapillêre sone elektroforese en serum vry ligte ketting analise in die diagnose van monoklonale gammopatie in pasiënt met HIV.

- Ek verklaar dat ek bogenoemde inligting gelees het of dit aan my verduidelik is en dat ek dit verstaan.
- Ek het die geleentheid gehad om vra te stel en dit is bevredigend beantwoord.
- Ek verstaan dat deelname an die projek vrywillig is en dat daar geen druk op my geplaas is om deel te neem nie en dat ek enige tyd mag onttrek.

Geteken te_____op_____

Handtekening van deelnemer: _____ Getuie:_____

Verklaring deur navorser:

Ek _____ verklaar dat ek die inligting in hierdie dokument aan bogenoemde pasient verduidelik, hom aangemoedig om vra te vra en is tevrede dat hy al die aspekte van die navorsingsprojek verstaan.

Geteken te_____op_____

Handtekening van navorser/klinikus:_____ Getuie:_____

Verklaring deur tolk (indien gebruik)

Ek _____ verklaar dat ek bogenoemde navorser bygestaan het om die inligting aan bogenoemde deelnemer te verduidelik in Xhosa.

- Ons het hom aangemoedig om vrae te vra en voldoende tyd gebruik om dit te antwoord.
- Ek het 'n feitilike korrekte weergawe oorgedra van dit wat aan my vertel is..
- Ek is tevrede dat die deelnemer die inhoud van die document ten volle verstaan.

Geteken te_____op_____

Handtekening van tolk:_____Getuie:_____

PARTICIPANT LEAFLET AND CONSENT FORM.

Title of research project**:**
Monoclonal gammopathy in patients with HIV: The prevalence and association with stage of disease, chronic antigen stimulation and the effect of HAART.

And

A comparison of the sensitivity and specificity of agarose gel electrophoresis, capillary zone electrophoresis and serum free light chain analysis in the diagnosis of monoclonal gammopathy in patients with HIV.

Reference nr:
Principle investigator: Dr A Zemlin
Address: Division of Chemical Pathology, NHLS, Tygerberg Hospital

- You are being invited to take part in a research project. Please take some time to read the information here, which will explain the details of this project.

- This study has been approved by the Committee for Human Research at Stellenbosch University and will be conducted according to the ethical guidelines and principles of the International Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medial Research Council (MRC) Ethical Guidelines for Research.

- The research project is about the following:
  The goal of the research project is to look at the antibody profile of patients with HIV infection on serum electrophoresis. Electrophoresis is a method used to separate different proteins. We are especially interested in the presence of monoclonal bands/peaks. That is the presence of a band on the serum electrophoresis that indicates a single type of antibody that is produced by clone of an antibody producing cells. The monoclonal peak is also found in patients who do not have HIV infection, where it often indicates the presence of lymphoma or multiple myeloma (a cancer of antibody producing cells) or precedes the development of it. A monoclonal peak is followed up carefully for the development of these conditions. Monoclonal peaks are more common in patients with HIV infection, but the significance is not clear. It may be the result of the immune response to the virus, or it may indicate a risk for developing lymphoma or multiple myeloma. We want to investigate the significance of these monoclonal peaks further to establish guidelines for their management and follow-up.

- The **same** specimens will also be analyzed with different methods (agarose gel electrophoresis, capillary zone electrophoresis and serum free light chains) to determine which method is the most sensitive and specific. This is important to make sure that the presence of peaks are not missed or on the other hand, unnecessary further investigations not performed. This data will also be published as a separate study.

- It involves the taking of a blood sample (10ml /2 teaspoons blood) and a urine sample (100ml/ half a cup) from you.

- If a monoclonal peak is present, you will be offered further investigations to rule out lymphoma or plasma cell tumours if necessary. This may involve X-rays, CT scan, bone marrow biopsy tests. This will be discussed with you and your consent obtained before proceeding with any further tests. The peak will also be followed up 6 monthly or yearly with a blood or urine sample.

- All patients with HIV infection that are followed up at the Tygerberg infectious Diseases Clinic are invited to participate in this study.

- The advantage of taking part in this study is that the presence of a monoclonal peak will be identified and followed up.

- The risks involved in participation in this research study includes the normal risks of blood taking which includes
  - pain at the site of blood sampling
  - bleeding and haematoma formation at the site of injury.

  If further follow up investigations are necessary, the procedure and risks will be explained and consent taken before proceeding.

- All information collected will be treated confidentially. It will be used in a publication and thesis, but the identity of participants will remain anonymous. Only the medical officers collecting the information and the researchers processing the information will have access to the information

- Participation is voluntary and you are free to decline to participate. You are also free to withdraw from the study at any point, even if you had agreed to take part. Participation in this study will not influence or change the treatment that you receive.

- You will not be paid to participate in the study, but there will be no costs involved for you, if you do participate.

- Any questions can be directed to the physician treating you. If more information is required, the investigators can be contacted at the above numbers:

Declaration by participant:

I _____agree to participate in the research project titeld:
Monoclonal gammopathy in patients with HIV: The prevalence and association with stage of disease, chronic antigen stimulation and the effect of HAART
and
A comparison of the sensitivity and specificity of agarose gel electrophoresis, capillary zone electrophoresis and serum free light chain analysis in the diagnosis of monoclonal gammopathy in patients with HIV.

- I declare that I read the above information or that it was explained to me and that I understand it.
- I had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is voluntary and I have not been pressurised to take part and that I may choose to leave the study at any time.

Signed at _____on_____

Signature of participant: _____ Witness:_____

Declaration by investigator:

I _____ declare that I explained the information in this document to the patient, encouraged him to ask questions and is satisfied that he understands all the aspects of this research project.

Signed at _____on_____

Signature of researcher/ clinician: _____Witness:_____

Declaration by translator (if used).

I _____ declare that I assisted the above named researcher to explain the information in Afrikaans / Xhosa (circle).

- We encouraged him to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has his/her questions satisfactorily answered.

Signed at _____on_____

Signature of translator:_____Witness: _____

APPENDIX 2

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot · your knowledge partner

28 April 2010

MAILED

Dr AE Zemlin
Dep of Chemical Pathology
9th Floor, NHLS
Tygerberg hospital
Francie van zijl drive
7500

Dear Dr Zemlin

"Monoclonal gammopathy in patients with HIV: the prevalence and association with stage of disease, chronic antigen stimulation and the effect of HAART."

ETHICS REFERENCE NO: N07/09/217

RE : AMENDMENT APPROVAL WITH STIPULATION

Your letter dated 16 April 2010 refers.

The Chairperson of the Health Research Ethics Committee approved the amended documentation in accordance with the authority given to him by the Committee.

Please state in the Participant Informed Consent form that these participants' blood will not be tested for HIV infection as part of the study.

Yours faithfully

MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
Fax: 021 931 3352

Fakulteit Gesondheidswetenskappe · Faculty of Health Sciences

Verbind tot Optimale Gesondheid · Committed to Optimal Health
Afdeling Navorsingsontwikkeling en -steun · Division of Research Development and Support
Posbus/PO Box 19063 · Tygerberg 7505 · Suid-Afrika/South Africa
Tel.: +27 21 938 9075 · Faks/Fax: +27 21 931 3352

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot · your knowledge partner

5 December 2007

Dr A Zemlin
Division of Chemical Pathology
Dept of Pathology

Dear Dr Zemlin

RESEARCH PROJECT: **"MONOCLONAL GAMMOPATHY IN PATIENTS WITH HIV: THE PREVALENCE AND ASSOCIATION WITH STAGE OF DISEASE, CHRONIC ANTIGEN STIMULATION AND THE EFFECT OF HAART"**

PROJECT NUMBER : **N07/09/217**

At a meeting of the Committee for Human Research that was held on 12 November 2007et was approved on condition that further information that was required, be submitted.

This information was supplied and the project was finally approved on 3 December 2007f one year from this date. This project is therefore now registered and you can proceed with the work. Please quote the above-mentioned project number in all further correspondence.

Please note that a progress report (obtainable on the website of our Division) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.

Patients participating in a research project in Tygerberg Hospital will not be treated free of charge as the Provincial Government of the Western Cape does not support research financially.

Due to heavy workload the nursing corps of the Tygerberg Hospital cannot offer comprehensive nursing care in research projects. It may therefore be expected of a research worker to arrange for private nursing care.

Yours faithfully

CJ VAN TONDER
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9207 / E-mail: cjvt@sun.ac.za

CJVT/pm

Fakulteit Gesondheidswetenskappe · Faculty of Health Sciences

Verbind tot Optimale Gesondheid · Committed to Optimal Health
Afdeling Navorsingsontwikkeling en -steun · Research Development and Support Division
Posbus/PO Box 19063 · Tygerberg 7505 · Suid-Afrika/South Africa
Tel: +27 21 938 9677 · Faks/Fax: +27 21 931 3352
E-pos/E-mail: rdsdorfo@sun.ac.za

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14 November 2011

MAILED

Dr AE Zemlin
Dep of Chemical Pathology
9th Floor, NHLS
Tygerberg hospital
Francie van zijl drive
7500

Dear Dr Zemlin

"Monoclonal gammopathy in patients with HIV: the prevalence and association with stage of disease, chronic antigen stimulation and the effect of HAART."

ETHICS REFERENCE NO: N07/09/217

RE : PROGRESS REPORT

At a meeting of the Health Research Ethics Committee that was held on 9 November 2011, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 9 November 2011

Expiry Date: 9 November 2012

Yours faithfully

MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
Fax: 021 931 3352

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APPENDIX 3

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM:
CONTROL GROUP

TITLE OF THE RESEARCH PROJECT

Free Light Chains in patients with HIV: establishing local reference ranges and their association with stage of disease, chronic antigen stimulation and the effect of HAART

PRINCIPAL INVESTIGATOR:
Dr Annalise Zemlin

CONTACT NUMBER:
Office: 021 – 938 4854 / 9384107

You are being invited to take part as a ***control (healthy individual)*** in a research project. Your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the Committee for Human Research at Stellenbosch University **and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.**

The project aims to determine the reference ranges of certain blood tests which will then be performed on patients infected with HIV to determine how they differ from the general population. For the study, we will need to test a small amount of your blood donated to the blood bank. *No extra blood will be collected*. No HIV test will be performed on this sample.

Free light chains are part of antibody molecules which are produced by the body in response to inflammation. We know that HIV may lead to a change in the production of antibodies, but have no information about the levels of free light chains in the HIV-infected patient. To be able to evaluate the relevance of the results, we have to compare it with the presence / absence of these free light chains in healthy individuals. We will therefore conduct a study at Tygerberg Hospital. Patients with advanced HIV infection have already enrolled in the study. In a comparative group, blood from 121 healthy blood donors is also needed to complete the study.

Declaration by participant

By signing below, I ……..…….. agree to take part in a laboratory based research study entitled:

Free Light Chains in patients with HIV: establishing local reference ranges and their association with stage of disease, chronic antigen stimulation and the effect of HAART

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (place) ……..…….. on (date) ……..…….. 2010.

…… ……

Signature of participant Signature of witness

Declaration by investigator

I (name) ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a translator.

Signed at (place) ……..…….. on (date) ……..…….. 2010.

…… ……

Signature of investigator Signature of witness

Decleration by Interpreter

I (name) ……..…… declare that:

- I have asisted the investigator *(name)*.....to explain the information in this document in English/Xhosa to *(name of participant)* .....
- I have encouraged him / her to ask questions and had ample time to answer any questions.
- I have given a factual version of what I was informed of
- I am satisfied that the participant fully understands the contents of this document and that his / her questions were answered satisfactory.

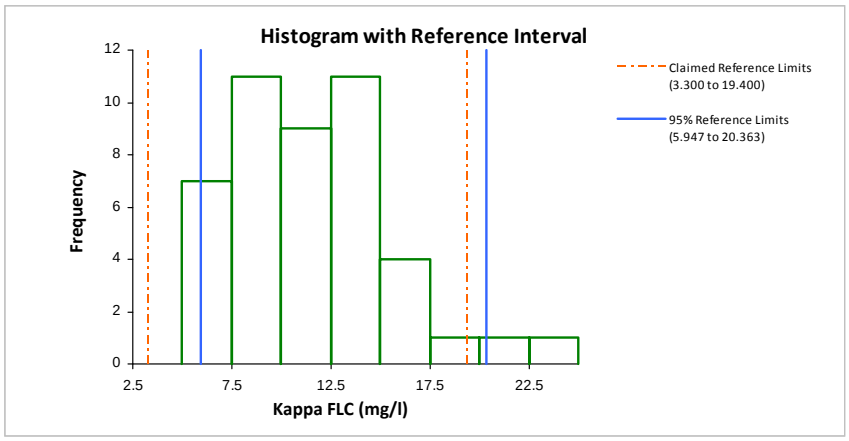
Signed at *(place)* ……..…….. on *(date)* ……..…….. 2010.

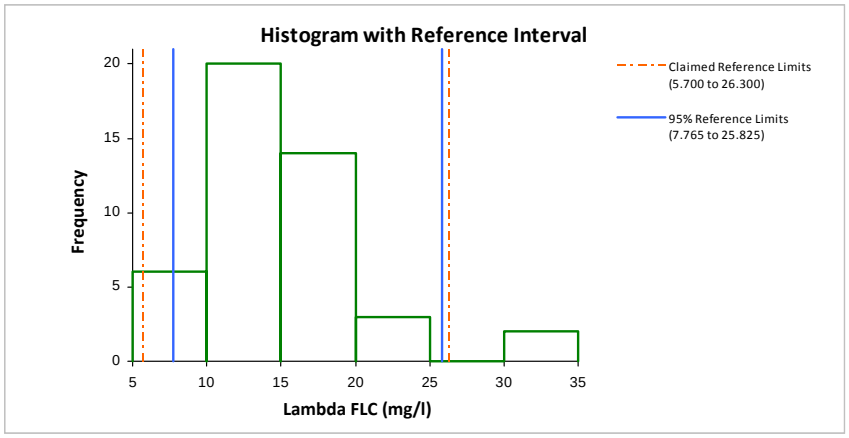
…… ……

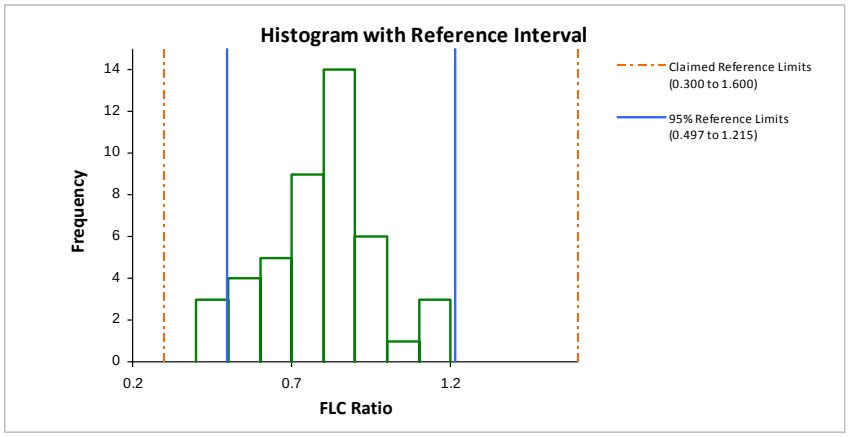
Signature of interpreter Signature of witness

APPENDIX 4

Graphs obtained for the 95% reference intervals on the Mixed Ancestry population.

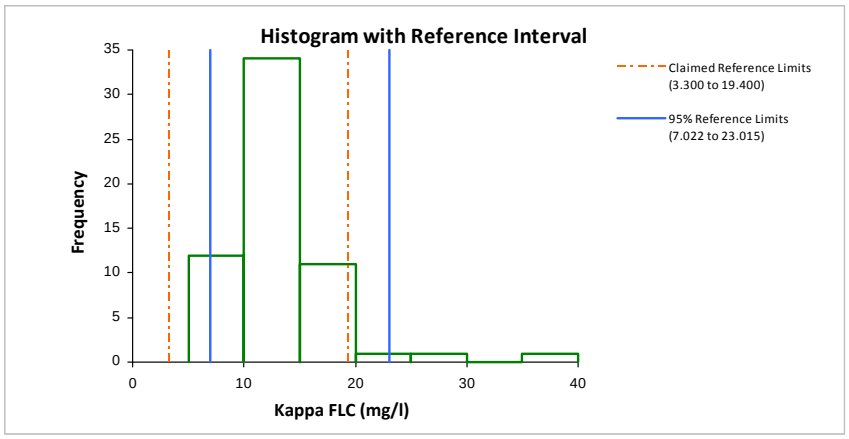


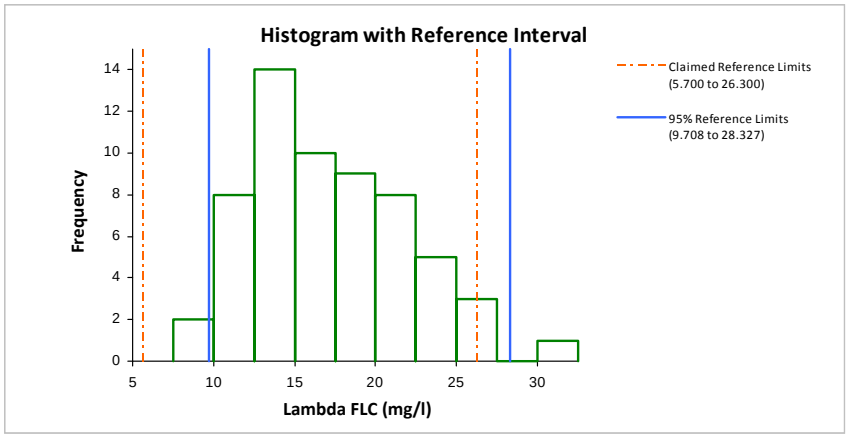


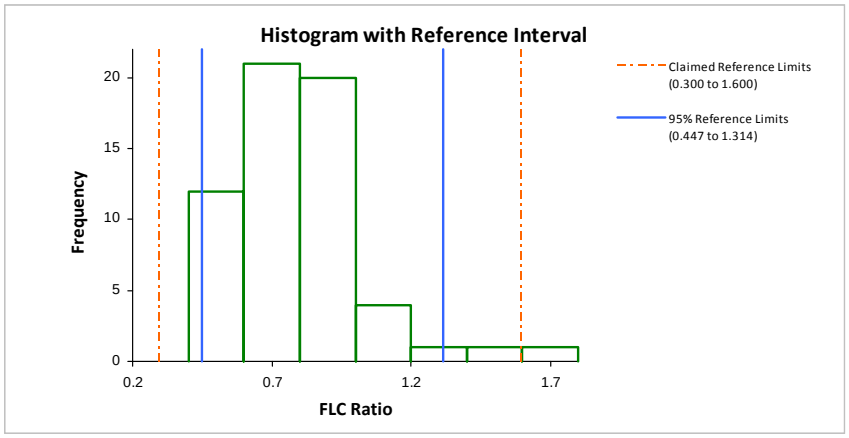


APPENDIX 5

Graphs obtained for the 95% reference intervals on the Black population.







APPENDIX 6

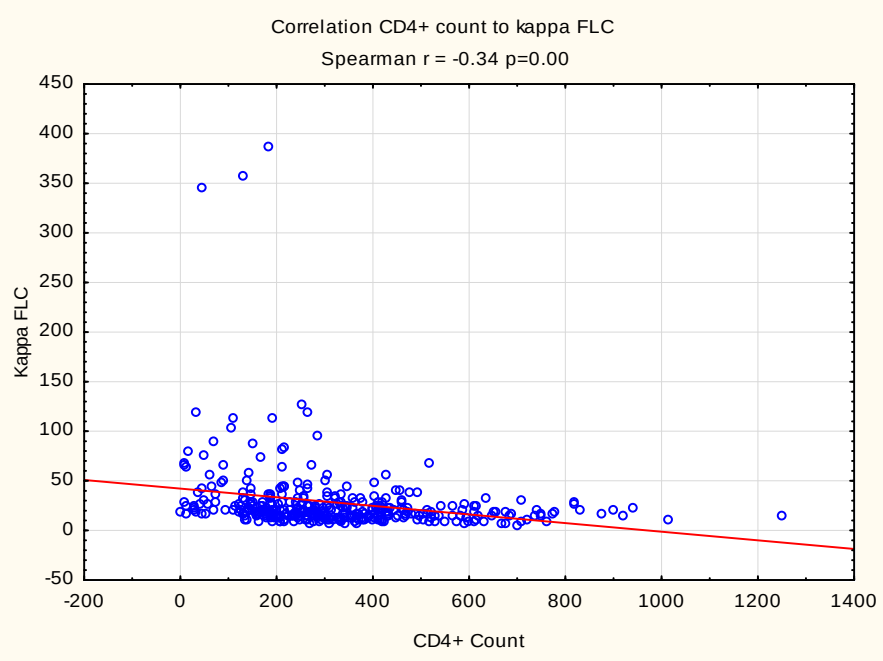


Figure 6.1 Correlation of $CD4^+$ count to κ FLC

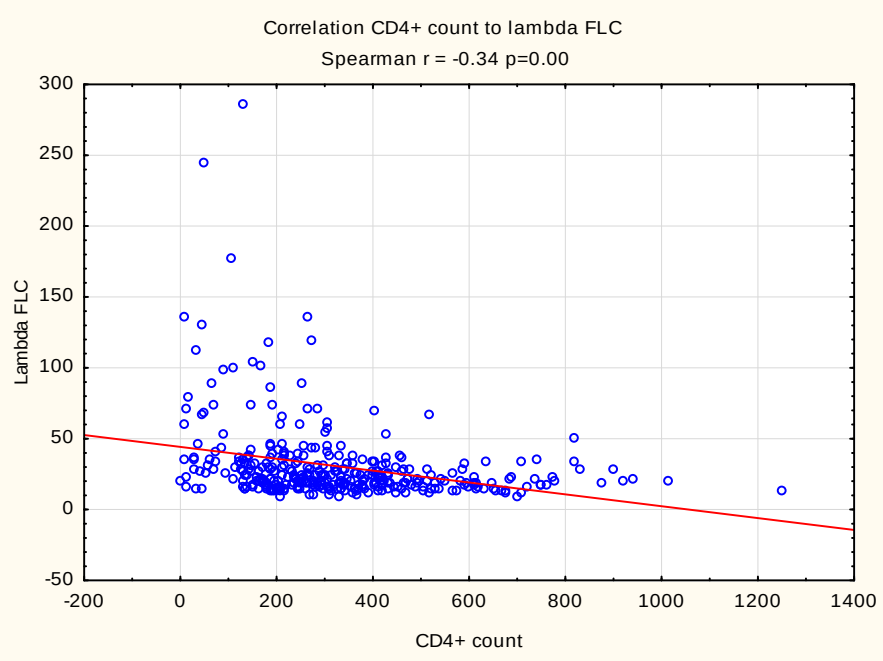


Figure 6.2 Correlation of $CD4^+$ count to λ FLC

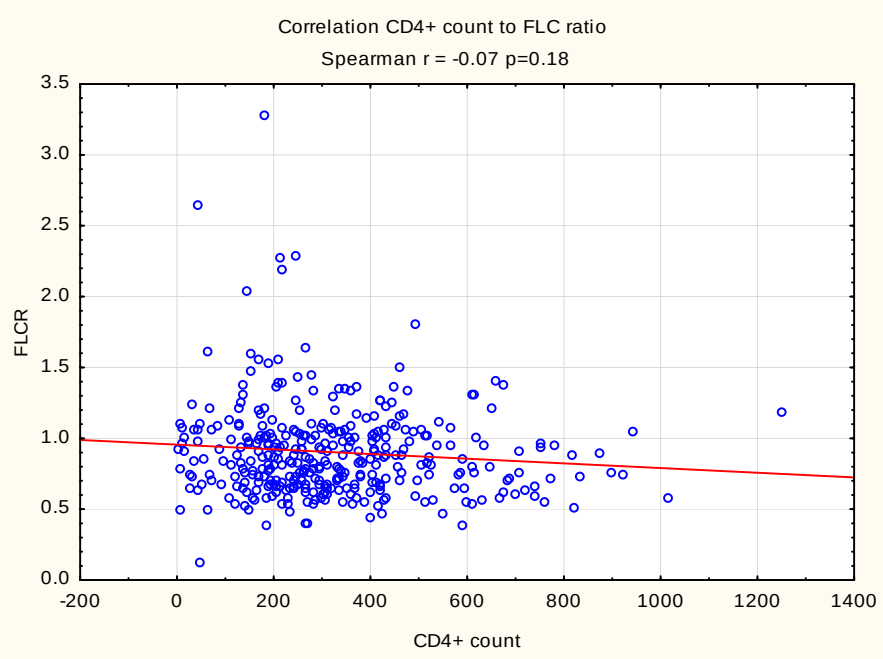


Figure 6.3 Correlation of $CD4^{+}$ count to FLC ratio

APPENDIX 7

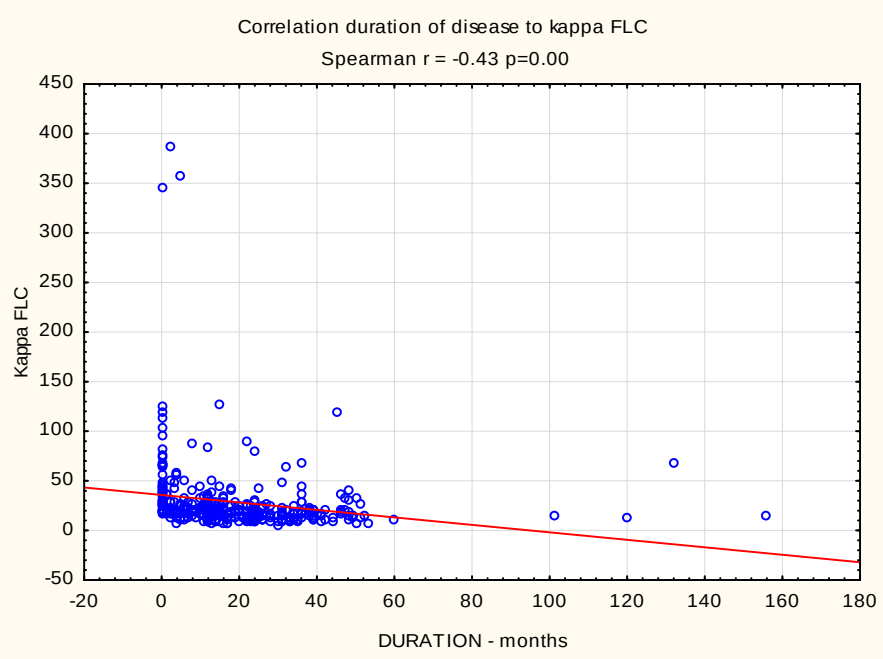


Figure 7.1 Correlation of duration of disease to κ FLC

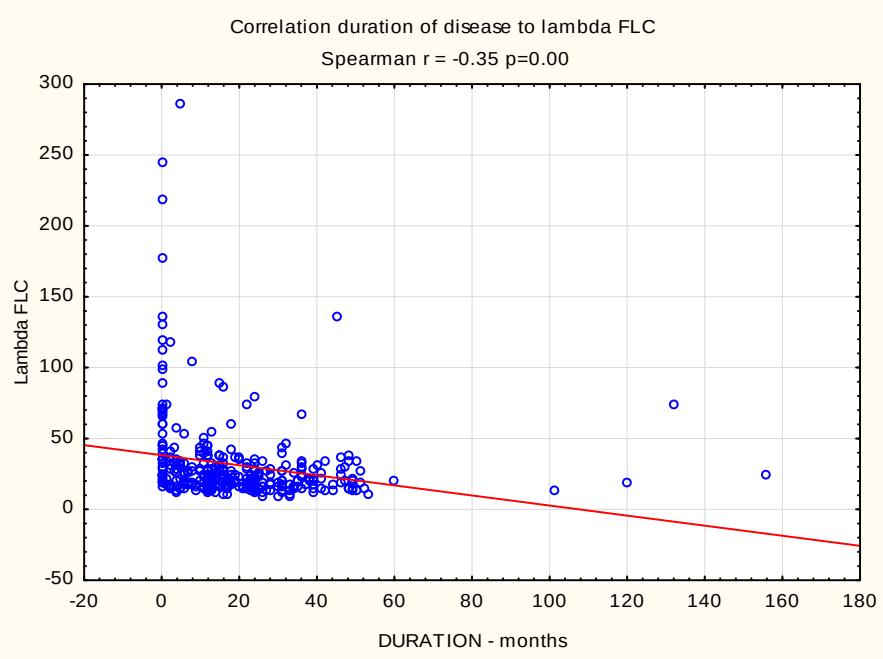


Figure 7.2 Correlation of duration of disease to λ FLC

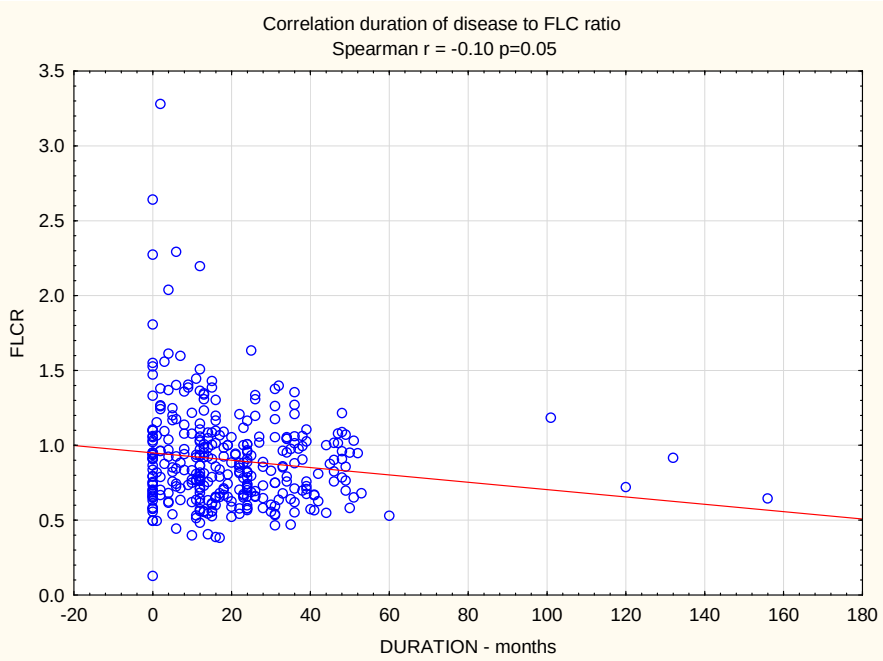


Figure 7.3 Correlation of duration of disease to FLC ratio

APPENDIX 8

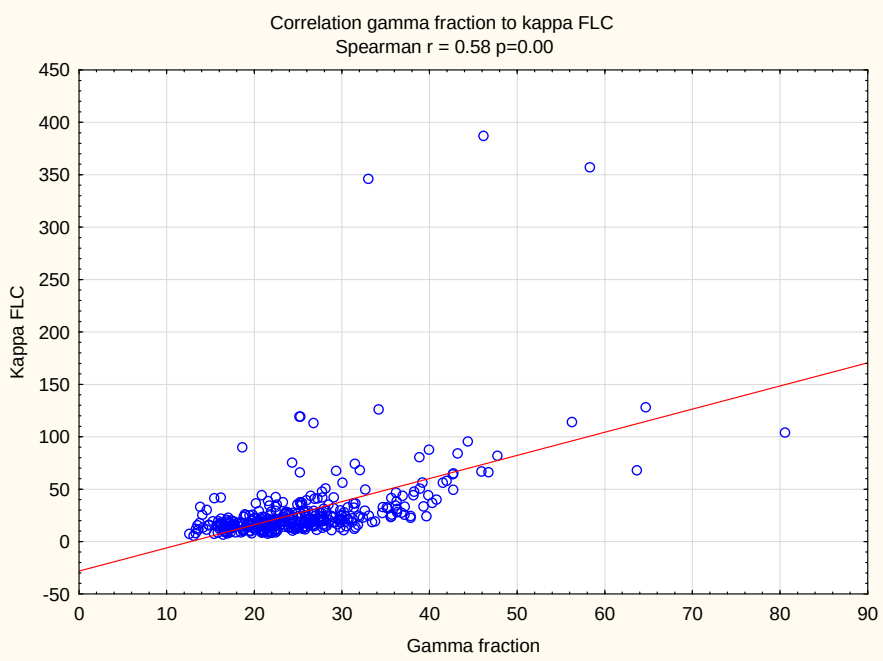


Figure 8.1 Correlation of gamma fraction to κ FLC

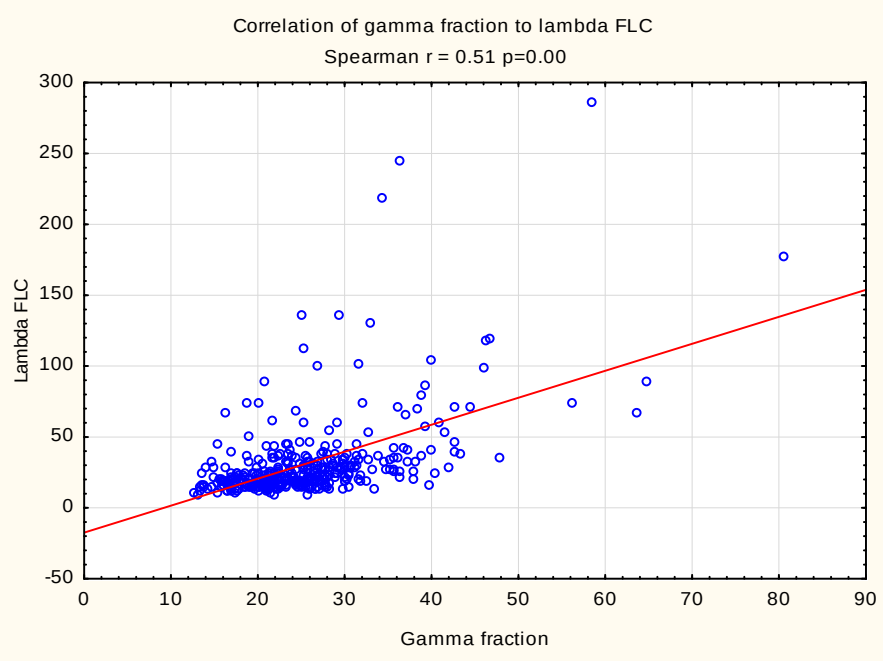


Figure 8.2 Correlation of gamma fraction to λ FLC

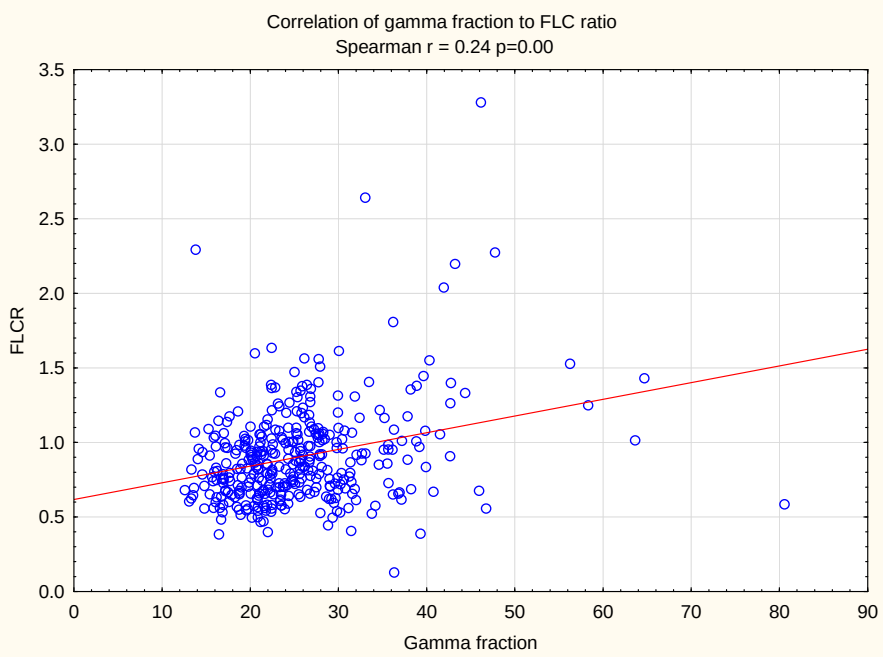


Figure 8.3 Correlation of gamma fraction to FLC ratio

APPENDIX 9

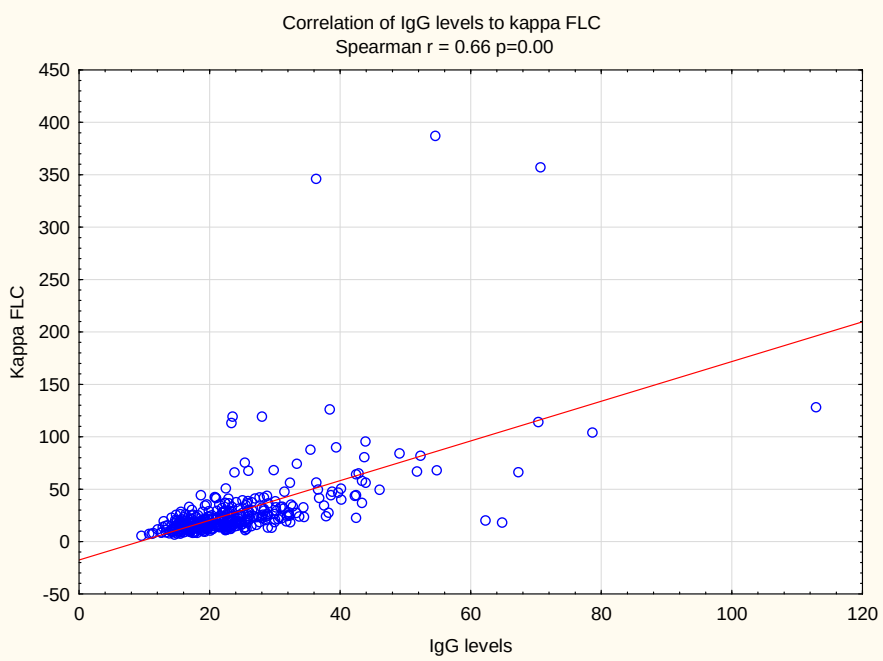


Figure 9.1 Correlation of IgG to κ FLC

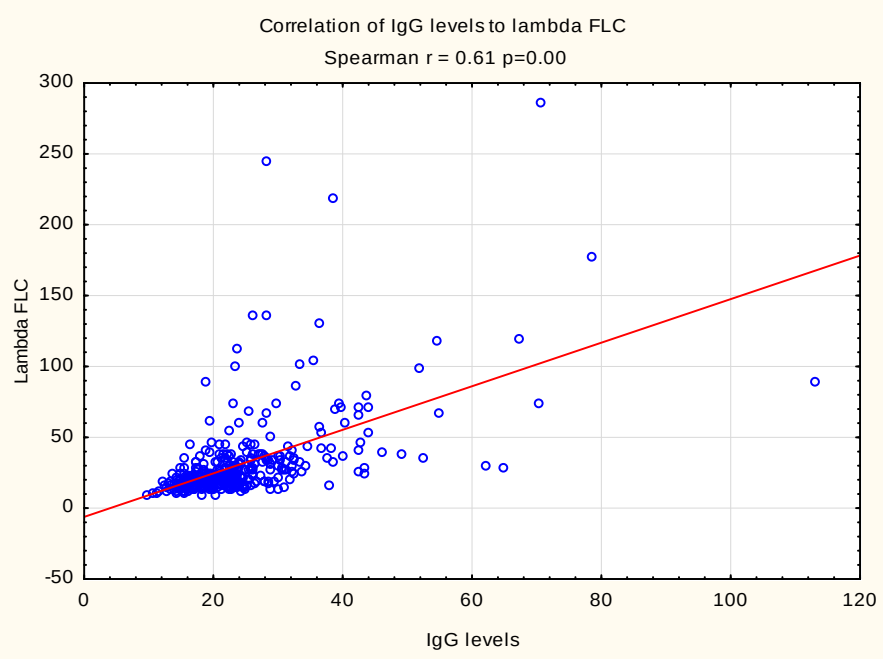


Figure 9.2 Correlation of IgG to λ FLC

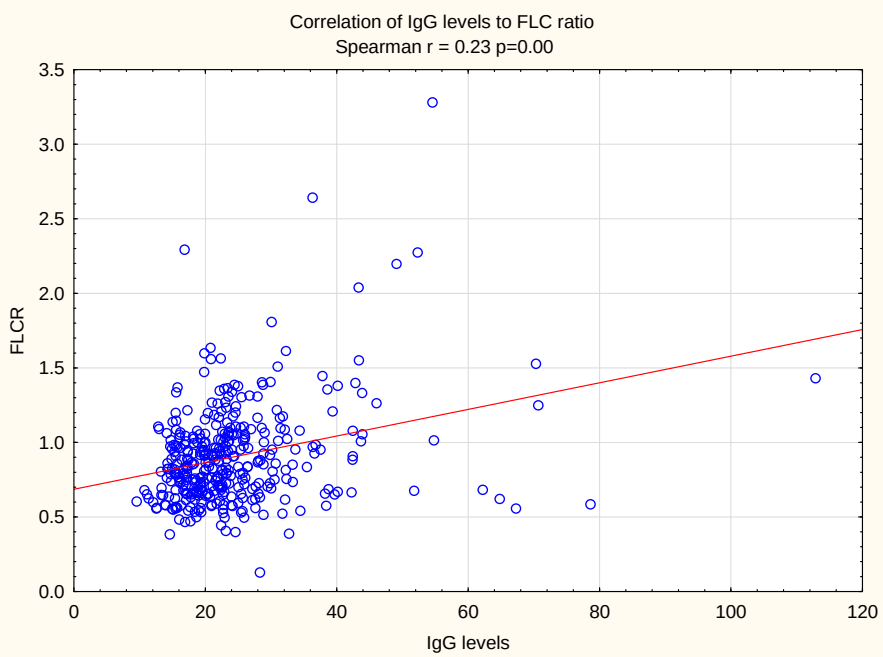


Figure 9.3 Correlation of IgG to FLC ratio

APPENDIX 10

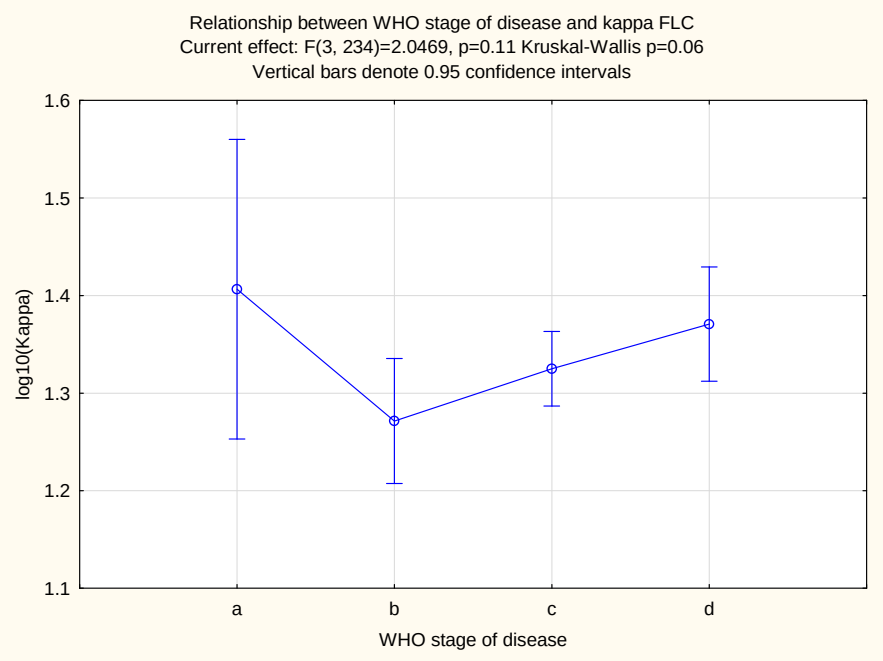


Figure 10.1 Relationship between WHO stage of disease and κ FLC

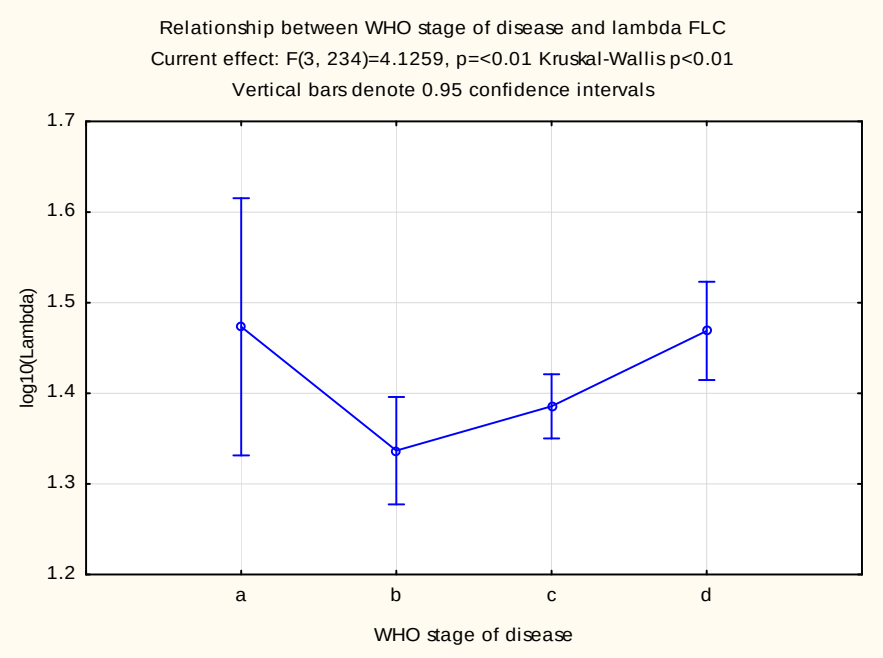


Figure 10.2 Relationship between WHO stage of disease and λ FLC

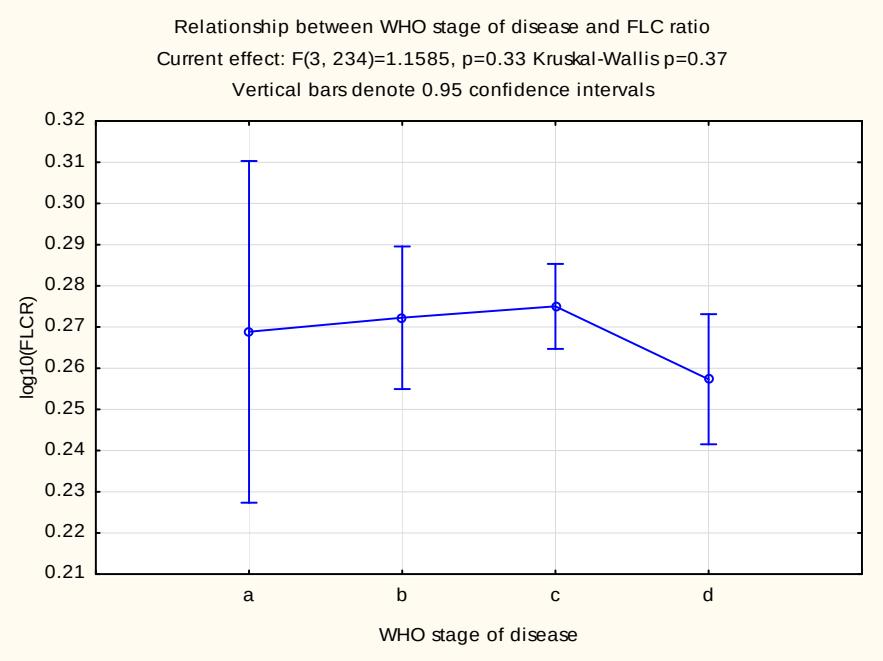


Figure 10.3 Relationship between WHO stage of disease and FLC ratio

APPENDIX 11

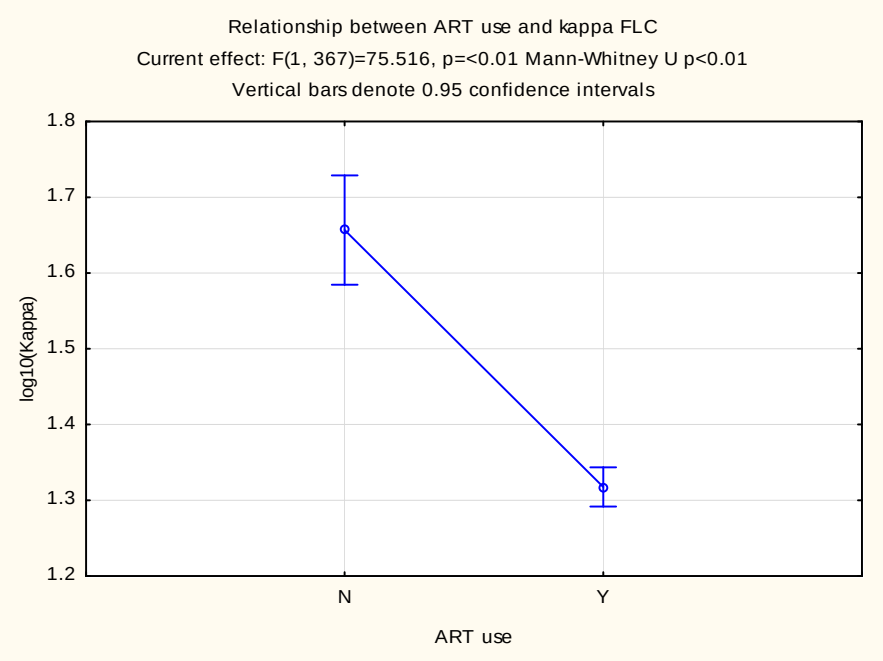


Figure 11.1 Relationship between ART use and κ FLC

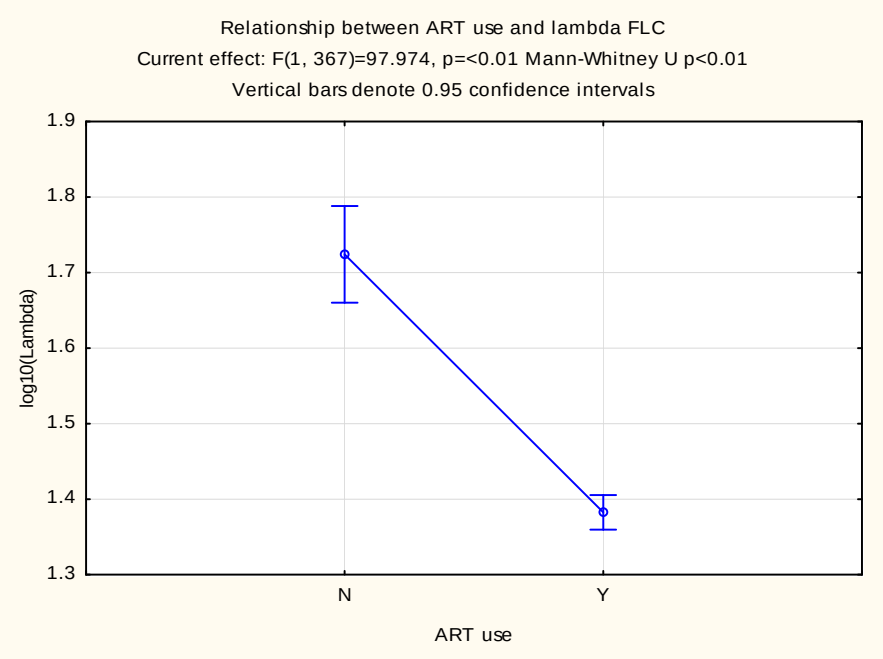


Figure 11.2 Relationship between ART use and λ FLC

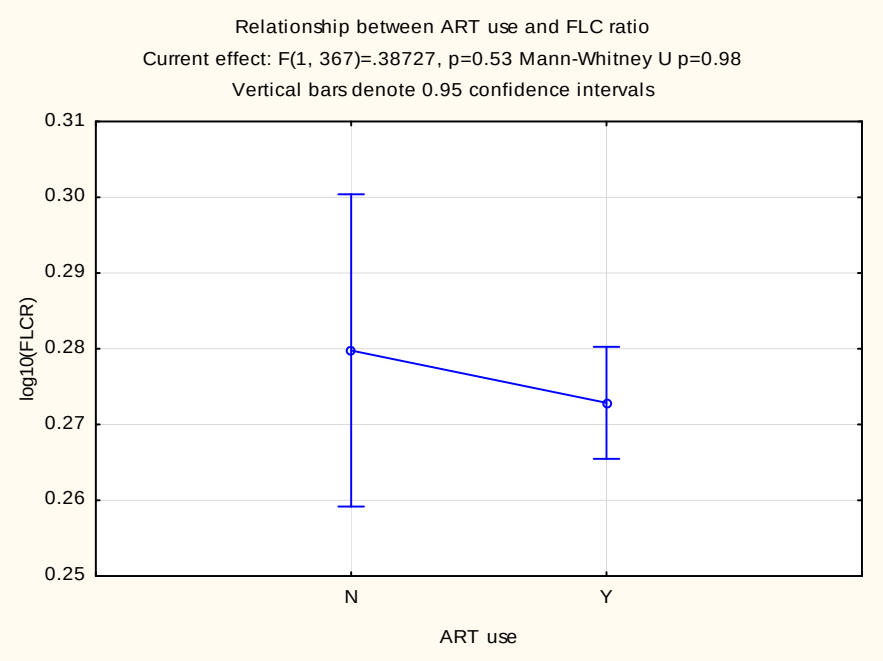


Figure 11.3 Relationship between ART use and FLC ratio

APPENDIX 12

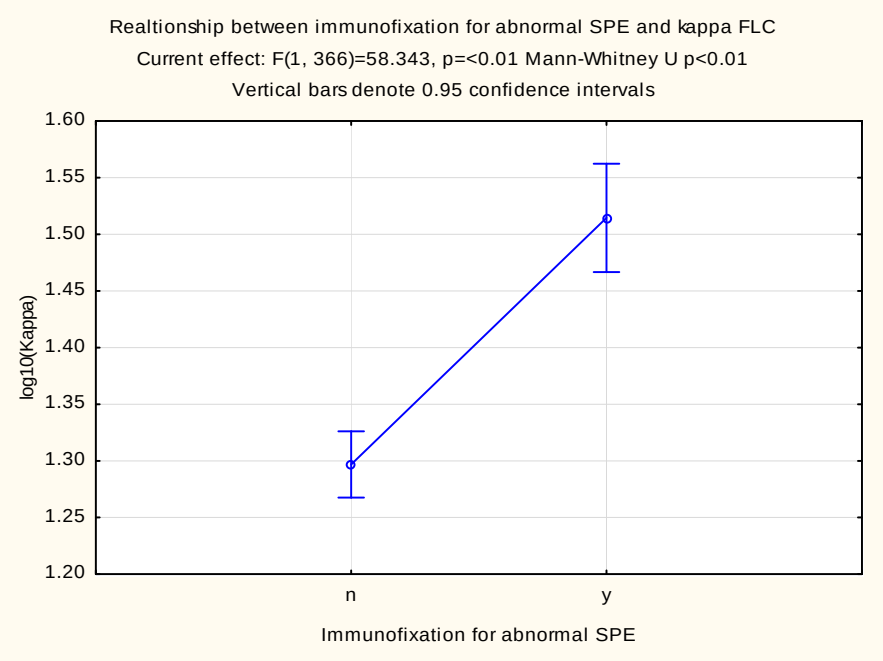


Figure 12.1 Relationship between immunofixation for abnormal serum protein electrophoresis and κ FLC

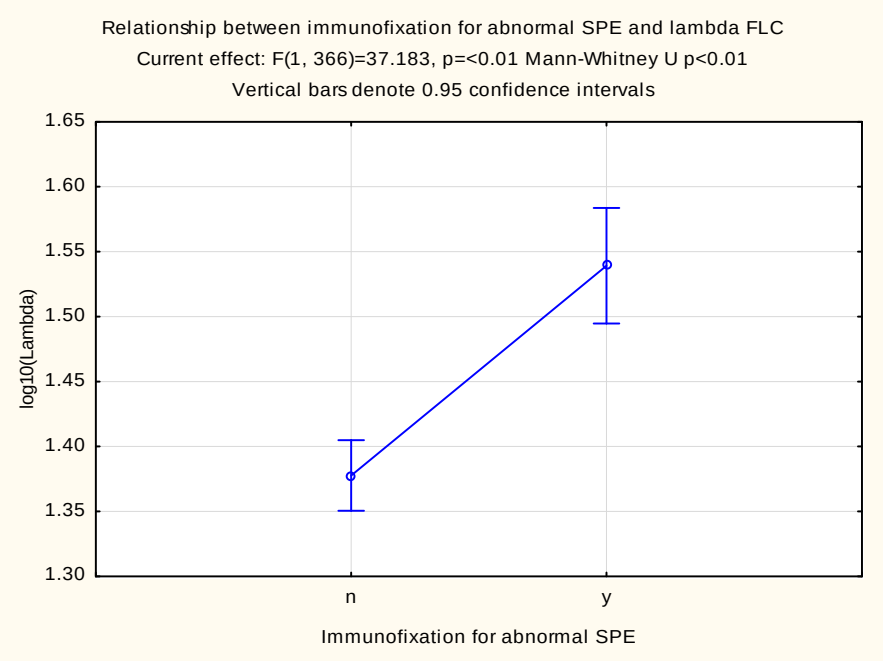


Figure 12.2 Relationship between immunofixation for abnormal serum protein electrophoresis and λ FLC

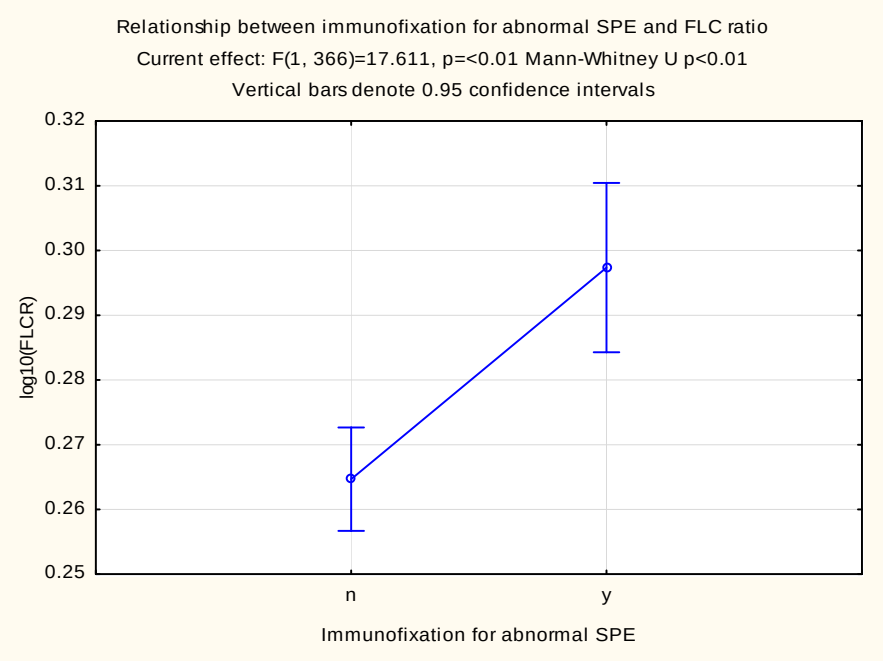


Figure 12.3 Relationship between immunofixation for abnormal serum protein electrophoresis and FLC ratio